

# **The role of free radicals in the pathogenesis of diabetic nephropathy**

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A thesis submitted in partial fulfillment of the requirements for the degree of  
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## DECLARATION

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work of which this is a record has been performed by myself, unless otherwise stated. All sources of information have been specifically acknowledged.



Anura Kalansooriya

April 2003

**DEDICATION**

To  
Gayathri  
Amanda & Amantha

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## ABSTRACT

The present study investigated the role of free radicals and associated enhanced oxidative stress in the pathogenesis of diabetic nephropathy. The study population comprised 40 diabetic subjects and 20 age- and gender-matched non-diabetic subjects. The diabetic subjects were further divided into two groups of 20 subjects, namely Group 1 (disease duration < 2 years) and Group 2 (disease duration 6-8 years).

Fasting plasma glucose, HbA<sub>1c</sub> and fructosamine levels, reflections of glycaemic control, were elevated in Group 2 compared to results in Group 1 which were in turn higher than control group values. Similar findings were observed with total cholesterol, triglycerides, LDL cholesterol and HDL cholesterol measurements.

Lipid peroxidation and antioxidant biochemical markers were also investigated in the study population. Urine TBARS were elevated in both Groups 1 and 2, consistent with the presence of enhanced lipid peroxidation in the diabetic state. The significant correlation between HbA<sub>1c</sub> levels and TBARS excretion also indicated an association between poor glycaemic control and increased lipid peroxidation. Furthermore, significant antioxidant depletion, assessed by measurements of vitamin E concentration, both erythrocyte and plasma glutathione peroxidase and plasma selenium concentrations was observed in Group 2 compared to results from Group 1 and control subjects.

Renal biomarker (creatinine, sialic acid and cystatin C in serum, and  $\alpha_1$  and  $\beta_2$ -microglobulin, AAP, NAG,  $\alpha$ GST and  $\pi$ GST in urine) studies revealed a significant

association between free radical formation and both early glomerular and renal tubular insult in the diabetic state. Indeed, elevated  $\pi$ GST enzymuria also indicated the presence of distal tubular insult in the diabetic state. Considering that hyperglycaemia, enzymuria, proteinuria, poor glycaemic control, dyslipidaemia and lipid peroxidation were most pronounced in Group 1 suggested that lipid peroxidation associated with oxidative damage and renal tubular insult may have accompanied or even preceded the onset/presence of microalbuminuria and renal structural alterations. Finally, NAG, AAP and particularly  $\alpha$ GST and  $\pi$ GST may be useful predictors of early renal insult in subjects with diabetes.

**ABBREVATIONS**

AAP	Alanine amino peptidase
ABTS	2, 2'-azino-di-(3-ethylbenzthiazoline-6-sulphonate)
ACR	Albumin: creatinine ratio
ADA	American diabetes Association
AGEs	Advanced glycation end products
AR	Aldose reductase
ATP	Adenosine triphosphate
BLM	Baso-lateral membrane
CAPD	Continuous ambulatory peritoneal dialysis
CML	N-ε-(carboxymethyl) lysine
CT	Collecting tubule
DAG	Diacylglycerol
DCCT	Diabetes Control and Complications Trial Group
DNA	Deoxyribonucleic acid
DT	Distal tubule
ECM	Extra cellular matrix
ESRD	End stage renal disease
GAD 65	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBM	Glomerular basement membrane
GCK	Glycolytic enzyme glucokinase
GFR	Glomerular filtration rate
GLUT4	Glucose transport 4
GOD	Glucose oxidase
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GST	Glutathione-S-transferase
HbA <sub>1c</sub>	Haemoglobin A <sub>1c</sub>
HDL	High-density lipoprotein
HLA	Human leukocyte antigens
HNE	4-hydroxynonenal

HNF-4 $\alpha$	Hepatocyte nuclear factor-4 alpha
HPLC	High performance liquid chromatography
HSP	Heparin sulphate proteoglycans
IA-2	Insulinoma associated antigen-2
IAA	Insulin auto-antibodies
IAPP	Islet amyloid polypeptide
ICA	Islet cell auto-antibodies
ICSA	Islet cell surface auto-antibodies
IDDM	Insulin dependent diabetes mellitus
IFG	Impaired fasting glycaemia
IFN $\gamma$	Interferon gamma
IGF-1	Insulin-like growth actor-1
IGT	Impaired glucose tolerance
IL-2	Interleukin-2
IPF-1	Insulin promoter factor -1
JNC	Joint National Committee
LADA	Latent autoimmune diabetes in adults
LDL	Low-density lipoprotein
MC	Mesangial cells
MDA	Malonldialdehyde
MES	2-morpholinoethane sulfonic acid
MODY	Maturity onset diabetes of the young
NAG	N-acetyl- $\beta$ -D-glucosaminidase
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide (oxidised form)
NADP <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitrotetrazolium-blue
NIDDM	Non- insulin dependent diabetes mellitus
NRP	Non-radical products
PKC	Protein kinase C
POD	Peroxidase
PT	Proximal tubule
PUFA	Polyunsaturated fatty acid



RAAS	Renin-angiotensin-aldosterone system
RBF	Renal blood flow
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TBM	Tubular basement membrane
TCRs	T cell receptors
TEAC	Trolox equivalent antioxidant capacity
TGF	Tubulo-glomerular feedback
TGF-β	Transforming growth factor beta
TINIA	Turbid metric inhibition immunoassay
TMB	3,3,5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha
TRIS	Tris (hydroxymethyl)-aminomethane
TSC	Trisodium citrate
UAE	Urinary albumin excretion
UKPDS	United Kingdom Prospective Diabetes Study Group
VLDL	very low-density lipoprotein
WHO	World Health Organisation

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# CHAPTER ONE

## INTRODUCTION

# **1. Introduction**

## **1.1 Diabetes mellitus**

Diabetes mellitus is a major global life-threatening disease with profound public health consequences. Although it has been centuries since diabetes was first recognised, it is still poorly understood and generally poorly managed. Consequently, the global prevalence of diabetes is predicted to rise from 135 million in 1995 to 300 million by 2025 (Dobson, 2000). Diabetes mellitus is defined as a metabolic disorder of multiple aetiology characterised by chronic hyperglycaemia with disturbances of carbohydrate; lipid and protein metabolism resulting from defects in insulin secretion, insulin action or a combination of both. It may present with characteristic symptoms such as polydipsia, polyuria, polyphagia, weight loss and the long-term effects of diabetes include progressive development of microvascular complications, particularly in the eye and kidney, and an increased frequency of macrovascular disease such as peripheral vascular and coronary heart disease (Bennett, 1989; WHO, 1999).

### **1.1.1 Historical review of diabetes mellitus**

Clinical features similar to diabetes were described 3000 years ago by the ancient Egyptians. The term "diabetes" was first coined by Araetus of Cappodocia (81-133AD) who introduced a clinical description of the disease, observing the increased urine flow, thirst hunger and weight loss, features which are instantly recognisable today. The sweet taste of urine in polyuric diabetics, which congregated ants was observed during the 5<sup>th</sup> and 6<sup>th</sup> century AD by ancient Indians (Susruta and Charuka) and these descriptions even

mention two forms of diabetes, one in older (adult onset), obese individuals and another in thin and younger subjects and this division predated the modern classification into type 2 and type 1 diabetes. Later, Thomas Willis rediscovered the sweetness of diabetic urine in 1675 (Papaspuros, 1964).

In 1776, Matthew Dobson in the UK made the crucial observation, confirmed that the sweetness in both urine and serum was due to sugar, and suggested that diabetes is a generalized disease. Cotunnus (McFarlane *et al*, 1997), who described the separation of a clot in heated diabetic urine, first discovered the presence of proteinuria in a diabetic patient in 1764. In 1797, attempts at treatment began on the basis of glycosuria and polyuria, John Rollo (McFarlane *et al*, 1997) performed an extended and meticulous metabolic study of a corpulent diabetic, and he demonstrated that the degree of glycosuria depended upon the type of food intake. Rollo also observed the smell of acetone on the breath of diabetics, presumably those in an advanced stage of type 1 diabetes, and he also observed cataracts in diabetics. However, in 1857, an important milestone in the history of diabetes was the establishment of the role of the liver in glycogenesis and the concept that diabetes was due to excess glucose production, which was discovered by Claude Bernard, and he also demonstrated links between the central nervous system dysfunction and the diabetic state (Papaspuros, 1964; Ekoe and Zimmet, 2001). In 1869, Paul Langerhans was the first to describe, pancreatic clusters of cells in teased preparations of pancreas which are now known as the “islets of Langerhans” and the role of the pancreas in the pathogenesis of diabetes was confirmed by Mering and Minkowski in



1889. Consequently, this discovery constituted the basis of insulin isolation and its clinical use by Banting and Best in 1921.

Complications of diabetes affecting the eyes, namely diabetic retinopathy, had been described before the discovery of insulin but it was a rarity because few diabetics lived long enough to develop retinopathy and glomerulosclerosis, which gradually progressed to renal failure and death and this condition was named the “Kimmelstiel-Wilson” nodules after its American and British co-discoverers in 1936. In addition, with longer survival of diabetic subjects, complications affecting the peripheral and later the autonomic, nervous system and gestational diabetes were described. However, at present, diabetes mellitus comprises a heterogeneous group of complex metabolic disorders of multiple aetiology and pathogenesis, which lead to a variety of complication and hyperglycaemia is the cardinal feature of these conditions and has therefore been used to define diabetes (Ekoe and Zimmet, 2001).

### **1.1.2 Diagnosis of diabetes mellitus**

As stated before diabetes mellitus is characterised as hyperglycemia and diagnosis is based on the presentation of subjects with the classical clinical features as defined earlier. However, diagnosis is more difficult in subjects who are asymptomatic and demonstrate marginally elevated glucose concentrations. Therefore, measurement of plasma glucose is the sole diagnostic criterion and the strategy is indirect as hyperglycemia reflects the consequence of the metabolic derangement, not the cause. However, until the underlying molecular pathophysiology of the disease is identified, plasma glucose concentrations are

likely to remain an essential diagnostic modality and the diagnosis of diabetes is established exclusively by the documentation of hyperglycemia, i.e. increased glucose concentrations in the plasma. However, widely accepted current (revised) diagnosis criteria have been introduced by the World Health Organization (WHO, 1999) and are shown in the Table 1.1.2.

**1.1.3 Classification of diabetes mellitus**

The clinical staging reflects that diabetes, regardless of its aetiology, progress through several clinical stages during its natural history. Furthermore, individual subjects may change from stage to stage in either direction. Subject who have, or who are developing, diabetes can be classified by stage according to the clinical characteristics, even in the absence of evidence concerning the underling aetiology. The first widely accepted classification of diabetes mellitus was published by the WHO Expert Committee in 1980 and subsequently updated in 1985. However, a recent WHO consultation document (1999) proposed a revised classification similar to that of the American Diabetes Association classification (ADA, 1997). Consequently, the new WHO classification encompassed both clinical stages and aetiological types of diabetes mellitus, mainly type 1, type 2 and other categories of hyperglycaemia, as suggested by Kuzuya and Matsuda, (1997) and are shown in the Table 1.1.3

**Table: 1.1.2 The new diagnostic criteria for the diagnosis of diabetes mellitus**  
(Values for diabetes mellitus and other categories of hyperglycaemia)

	Glucose concentration (mmol /L)			
	Whole blood		Plasma	
	Venous	Capillary	Venous	Capillary
<b>Diabetes mellitus</b>				
Fasting or	≥6.1	≥6.1	≥7.0	≥7.0
2-h post-glucose load	≥10.0	≥11.1	≥11.1	≥12.2
or both				
<b>Impaired glucose tolerance (IGT)</b>				
Fasting (if measured) and	<6.1 and	<6.1 and	<7.0 and	<7.0 and
2-h post-glucose load	≥6.7 and <10.0	≥7.8 and <11.1	≥7.8 and 11.1	≥8.9 and <12.2
<b>Impaired fasting glycaemia (IFG)</b>				
Fasting	≥5.6 and <6.1	≥5.6 and <6.1	≥6.1 and <7.0	≥6.1 and <7.0
and (if measured) 2-h	<6.7	<7.8	<7.8	<8.9
post-glucose load				

The glucose load refers to 75 g of anhydrous glucose (or monohydrate equivalent) dissolved in 250-300 ml of water and consumed over the course of 5 min. For clinical purposes, the diagnosis of diabetes should always be confirmed by repeating the test on another day unless there is unequivocal hyperglycaemia with acute metabolic decompensation.

(Report of World Health Organisation 1999)



**Table: 1.1.3 Clinical and aetiological Classification of Disorder of Glycaemia**

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Type 1 diabetes mellitus:	( $\beta$ -cell destruction, usually leading to absolute insulin deficiency). Autoimmune diabetes mellitus Idiopathic diabetes mellitus
Type 2 diabetes mellitus:	(May range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance).
Other specific types:	Genetic defects of $\beta$ -cell function Genetic defects in insulin action Disease of the exocrine pancreas Endocrineopathies Drug-or chemical-induced Infections Uncommon forms of immune-mediated diabetes Other genetic syndromes sometimes associated with diabetes
Impaired Glucose Tolerance	
Gestational diabetes mellitus	

---

Adopted from Report of a WHO Consultation Document (1999)

Type 1 diabetes mellitus, previously known as insulin dependent diabetes mellitus (IDDM) or juvenile-onset diabetes mellitus, results from autoimmune destruction of the  $\beta$ -cells of the pancreas, rendering the pancreas unable to synthesize and secrete insulin. Therefore, type 1 diabetics are entirely dependent on endogenous insulin for their survival.

Type 2 diabetes mellitus, previously encompassed non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, resulting from a combination of insulin resistance and inadequate insulin secretion. Type 2 diabetes can be controlled by various combinations of dietary modifications, changing lifestyle, increase physical activity and certain forms of oral hypoglycaemic agent without depending on exogenous insulin. However, some do require some insulin therapy at diagnosis and subsequently oral hypoglycaemic agents or dietary modifications. Other types of diabetes are rare as indicated in the Table 1.1.3

#### **1.1.4 Overview of Pathogenesis of diabetes mellitus**

##### **1.1.4.1 Pathogenesis of type 1 diabetes mellitus**

Type 1 diabetes is a chronic disorder resulting from the autoimmune mediated destruction of the  $\beta$ -cells of the pancreas (WHO, 1999). The rate of destruction is quite variable, a rapidly progressive form is commonly observed in children and a slow progressive form generally occurs in adults, which is referred to as latent autoimmune diabetes in adults (LADA). Type 1 diabetes commonly produces subjective and objective signs or biochemical changes within a short time duration. The major metabolic derangements are

marked hyperglycaemia, excessive ketonaemia, and ketonuria resulting in a life threatening metabolic acidosis commonly referred to as diabetic ketoacidosis (DKA), observed commonly in type 1 diabetics (Foster and McGarry, 1983).

Type 1 diabetes can be classified, according to presenting clinical characteristics as well as by immune and genetic markers. About 80% of subjects with type 1 diabetes have specific HLA phenotypes associated detectable autoantibodies such as specific islet cell autoantibodies (ICA) (Botazo *et al*, 1974), islet cell surface autoantibodies (ICSA) (Lernmark, 2001), insulin autoantibodies (IAA) (Palmer, 1983), glutamic acid decarboxylase (GAD65) (Karlsen, 1992), and insulinoma associated antigen-2 (IA-2) (Payton, 1995) which have all been recognized as dynamic pathogenetic markers of an ongoing disease process.

The process of  $\beta$ -cell autoimmunity appears to be initiated long before the symptoms of hyperglycaemia are present but the clinical onset of disease is not apparent until there is a major loss (80% to 90%) of islet  $\beta$ -cell function. Several studies have also demonstrated a direct association between the clinical onset of disease and acute viral infections (Yoon, 1995; Cahill *et al*, 1981). In animal cell culture, other studies have demonstrated that Coxsackie B virus infection is probably associated with virus replication in the  $\beta$ -cells followed by the formation of GAD65 antibodies (Yoon *et al*, 1995; Gerling *et al*, 1991, Hou *et al*, 1993). Apart from Coxsackie B virus, in human studies, different virus species studies include congenital rubella (McIntosh and Menser, 1992), cytomegalovirus (Ward *et al*, 1995), retroviruses and Epstein-Barr virus (Krieg *et al*, 1992) have been implicated



in the initiation and progression of  $\beta$ -cell destruction. Although, several viral-mediated pathomechanism of  $\beta$ -cell destruction have been proposed using both different animal models of type 1 diabetes and in human studies, the exact mechanisms are not clearly defined for most viruses in the pathogenic process.

Early exposure to cow's milk proteins is also associated with an increased risk of childhood diabetes. Molecular mimicry between bovine serum albumin and ICA69, another putative  $\beta$ -cell autoantigen, has been suggested (Pietropaolo *et al*, 1993). Children fed cow's milk formula had higher titers of IgG antibodies to bovine insulin that cross reacted with human insulin, and the presence of these antibodies correlated with the presence of human IAA (Vaarala *et al*, 1998). These findings may explain the high incidence of IAA in young children and raise the possibility that sensitization to bovine insulin could prime immune system auto aggression towards  $\beta$ -cells in subjects who are genetically predisposed (Vaarala *et al*, 1998).

The pathogenetic process is probably the same before and after the clinical diagnosis of diabetes and the rate of  $\beta$ -cell destruction also appears to be influenced by human leukocyte antigens (HLA). The HLA DQB1\*0302-A1\*0301/DQB1\*0201-A1\*0501 genotype appears to be associated with accelerated  $\beta$ -cell destruction and a more rapid progression to clinical onset of type 1 diabetes (Graham *et al*, 1999). HLA molecules are important determinants in regulating the immune response in humans; the susceptibility to type 1 diabetes is speculated to be conferred by the functional importance of these molecules in the restriction of the immune response. Furthermore, the HLA class II molecules, the latter which are strongly associated with type 1 diabetes specifically bind

short peptides or epitopes. These are recognised by specific T-cell receptors (TCRs) on CD4<sup>+</sup> T lymphocytes, which is the signal to initiate the development of cytotoxic T lymphocytes and B-lymphocytes, which produce antibodies to the antigen. This entire process is affected by a number of cytokines, such as interferon gamma (IFN  $\gamma$ ), tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-2 (IL-2) (Lernmark, 2001). However, recent studies have revealed that these cytokines (IFN  $\gamma$ , TNF- $\alpha$ ) are associated with an increased production of NO due to formation of free radicals and reactive oxygen intermediates (Thomas and Kay, 2000), and the elevated concentration of NO leads to DNA damage and programmed cell death (apoptosis) by as yet poorly defined mechanisms (Mathis *et al*, 2001). Furthermore, other reactive oxygen species such as hydrogen peroxide (Rabinovitch *et al*, 1992), and peroxynitrite might also be involved in  $\beta$ -cell destruction during the development diabetic states. Over expression of antioxidants such as catalase (Lortz *et al*, 2000), manganese superoxide dismutase and glutathione peroxidase have an important influence on cytokine-mediated killing *in vitro* or on diabetes development as demonstrated in animal models (Lortz *et al*, 2000).

#### **1.1.4.2. Pathogenesis of type 2 diabetes mellitus**

Type 2 diabetes is a heterogeneous syndrome in which hyperglycaemia resulting both an impaired insulin secretory response to glucose and decreased effectiveness in stimulating glucose uptake by skeletal muscle and in restraining hepatic glucose production, is present (DeFronzo, 1997; WHO, 1999). The primary biochemical events leading to chronic hyperglycaemia are still controversial (Ferrannini, 1998) and type 2 diabetes probably results from a complex interaction of genetic and environmental factors influencing characteristic features relevant to the diabetic phenotype such as insulin



action, insulin secretion,  $\beta$ -cell mass, fat distribution and obesity (Velho and Frogual, 2001). However, longitudinal studies suggest that abnormalities in both insulin action and insulin secretion occur early in the pathogenesis of type 2 diabetes (Ferrannini, 1998).

Primary defects in pancreatic  $\beta$ -cells have been recognised in maturity onset diabetes of the young (MODY), a rare form of diabetes resulting from gene mutation. MODY is a clinically heterogeneous syndrome characterized by non-ketotic diabetes, an autosomal dominant mode of inheritance and onset usually before 25 years of age, frequently in childhood or adolescence (Bell and Polonsky, 2001). Recently, different types of MODY genes have been identified that encode the glycolytic enzyme glucokinase (GCK) and transcription factors include hepatocyte nuclear factor (HNF)-4 $\alpha$ /MODY1, glucokinase/MODY2, HNF-1 $\alpha$ /MODY3, insulin promoter factor (IPF)-1/MODY4, HNF-1 $\beta$ /MODY5, neurogenic differentiation (NeuroD1)/MODY6 and Islet (Isl)-1/MODY7 (Bell and Polonsky, 2001; Iwasaki, 2001). The defect in GCK is a stable defect of glucose sensing, whereas the HNF-1 $\alpha$  mutation causes a progressive defect that alters  $\beta$ -cell insulin secretion rather than the sensing of glucose. (Pearson *et al*, 2001).

Insulin resistance is a major risk factor for the pathogenesis of type 2 diabetes and the insulin resistance syndrome (syndrome X) (Reaven, 1995). Insulin resistance is a metabolic state in which a normal concentration of insulin produces a less than normal biological response. The clinical manifestations of insulin resistance, glucose intolerance and hyperinsulinemia, are the consequence of the inability of insulin to stimulate glucose uptake in such target tissues as muscle and adipose tissues. Evidence exists that insulin resistance reduces glucose transport activity, the rate-limiting step for glucose uptake

(Garvey *et al*, 1998). Insulin stimulates glucose transport via translocation of GLUT4 glucose transporter proteins from an intracellular depot to the cell surface. Hence, GLUT4 depletion appears to be the major cause of insulin resistance in adipose tissue because of the profound degree of GLUT4 loss and its central role in transport stimulation (Garvey *et al*, 1998). Furthermore, recent studies have demonstrated that insulin receptor tyrosine kinase activity, together with downstream signalling events for example, insulin receptor substrate 1 (IRS-1) phosphorylation, phosphatidylinositol-3 kinase (PI-3-kinase) activation, are reduced in skeletal muscle (Goodyear *et al*, 1995). Thus, one potential cause of human insulin resistance is a defect in insulin signalling that impairs GLUT4 translocation in muscle (Garvey *et al*, 1998). Although insulin resistance involves decreased glucose transport activity in skeletal muscle, its molecular basis again remains unclear.

Obesity is the most common cause of insulin resistance in humans (Cavaghan *et al*, 2000). In obese subjects, insulin concentrations typically increase to maintain normal glucose tolerance. Basal and total daily 24h rates of insulin secretion are three to four times higher in obese insulin resistant subjects than in lean controls (Cavaghan *et al*, 2000). There is an inverse relationship between insulin sensitivity and insulin secretion, which is indicative of  $\beta$ -cell compensation for insulin resistance. As insulin resistance increases, the early first-phase insulin response must increase proportionately to maintain normal glucose tolerance. However, in type 2 diabetes, there is an overall reduction in insulin secretion rate as the  $\beta$ -cell can no longer secrete sufficient insulin to maintain normal blood glucose concentrations. (Bell and Polonsky, 2001).



The islet amyloid deposition is a pathological feature of the pancreas in type 2 diabetes that is associated with reduced  $\beta$ -cell mass and insulin secretion (Kahn *et al*, 1999). The unique peptide component of islet amyloid is islet amyloid polypeptide (IAPP) or amylin, a normal secretory product of the islet  $\beta$ -cell released with insulin in response to nutrient stimuli such as glucose and amino acids (Kahn *et al*, 1990). The human IAPP (hIAPP) molecule contains a specific sequence of amino acids rendering it capable of forming amyloid fibrils. The association of amyloid deposits and relative hyperproinsulinaemia, suggests that fibril formation begins during impaired glucose tolerance after other factors cause the initial defects in early insulin secretion and insulin action in type 2 diabetes (Porte and Kahn, 2001).

#### **1.1.5. Biochemical basis of diabetic complications**

All forms of diabetes are typified by hyperglycaemia, a relative or absolute deficiency of insulin, and the development of diabetic-specific complications. A diagnosis of diabetes immediately increases the risk of developing various diabetic-specific clinical complications that are largely irreversible and due to microvascular and macrovascular disease. Large prospective clinical studies demonstrated a strong relationship between glycaemic control and the development of microvascular complications in both type 1 and type 2 diabetes (DCCT, 1993; UKPDS, 1998). Furthermore, hyperglycaemia and insulin resistance also both play important roles in the pathogenesis of macrovascular disease (UKPDS, 1998). As a consequence of its underlying microvascular pathology, diabetes is a leading cause of blindness (retinopathy), end stage renal disease

(nephropathy) and a variety of debilitating neuropathies. Diabetes is also associated with accelerated atherosclerotic macrovascular disease affecting arteries, brain and lower extremities, higher risk of myocardial infarction, stroke and limb amputation (Brownlee, 2001). The 'glucose hypothesis' attributed the microvascular and neuropathic complications of diabetes to chronic hyperglycaemia and it postulates that hyperglycaemia precipitates these complications. Clinical and epidemiological data from human studies suggest that the magnitude and duration of hyperglycaemia in diabetes are strongly associated with the severity of microvascular complications (Engerman and Kern, 1987; Danne *et al*, 1994) but genetic and environmental factors may also be associated with the development and progression of these complications.

Diabetic retinopathy primarily affects the retinal blood vessels, which may progress through different stages of retinopathy including background, preproliferative, proliferative, advanced and maculopathy according to the features demonstrated on ophthalmoscopy. Cataract is also a common cause of blindness in diabetics due to non-enzymatic glycation of lens proteins and the accumulation of sorbitol in the diabetic lens as a consequence of increased polyol pathway activity. The clinical manifestations of neuropathy in subjects with type 1 and type 2 diabetes can be severe. A peripheral, symmetric sensorimotor neuropathy is the most common form of diabetic neuropathy that affects the long nerves, whereas other forms include cranial and peripheral motor neuropathies and autonomic neuropathy, which is asymptomatic (Nathan, 1993). As a consequence of hyperglycaemia, the late complications of diabetes represent in large part, microvascular dysfunction (Tooke, 1995) and diabetic-specific complications in the retina, glomerulus and vasa nervorum have similar pathophysiological features



(Brownlee, 2001). At an early stage in the course of diabetes, intracellular hyperglycaemia is associated with abnormalities in blood flow and increased vascular permeability (Brownlee, 2001). This reflects decreased activity of vasodilators such as nitric oxide, increased activity of vasoconstrictors, for example angiotensin II, and endothelin-1, and elaboration of permeability factors such as vascular endothelial growth factor (Ebara *et al*, 2000). An important link between the polyol pathway and non-enzymatic glycation is supported by the ability of aldose reductase inhibitors to stimulate the action of amino guanidine (a drug used on controlling AGEs formation) and the fact that both increased polyol pathway activity and non-enzymatic glycation process increase the rate of free radical production (Took, 1995). This may ultimately lead to compound endothelial dysfunction. Furthermore, micro vascular cell loss occurs, in part because of apoptosis, and there is progressive capillary occlusion caused by both extra cellular matrix overproduction induced by growth factor including transforming growth factor- $\beta$  (TGF- $\beta$ ). In contrast, hyperglycemia may change the function of endothelial and neuronal cells resulting in edema, ischemia and hypoxia-induced revascularization in the retina, essential matrix expansion and glomerulosclerosis in the kidney and multifocal axonal degeneration in peripheral nerves (Brownlee, 2001).

In diabetic macrovascular disease, it has been postulated that both hyperglycaemia and insulin resistance may play a significant role. In diabetic arteries, endothelial dysfunction may involve both insulin resistance and hyperglycaemia. Furthermore, in diabetic dyslipidaemia, hyperglycaemia increases atherogenic cholesterol-enriched apolipoprotein B-containing remnant particles by reducing expression of the heparin sulphate proteoglycan on hepatocytes (Ebara *et al*, 2000) and the associations of atherosclerosis

and atherosclerosis risk factors with glycaemia have been demonstrated over a wide range of glucose tolerance, from normal to diabetic (Brownlee, 2001).

In contrast, the UK Prospective Diabetes Study (UKPDS, 1998) and the Diabetes Control and Complications Trial (DCCT, 1993) Research Group emphasized that diabetic complications are common, frequently present by the time of diagnosis, and significantly affect the quality of life. Therefore, the preclinical stages of retinopathy, nephropathy and neuropathy might also be appropriate targets for screening and diagnosis (Nathan, 1993) of diabetic complications. However, it is now well established that intensive therapy to improve glycaemic control reduces the risk of microvascular complications in general and diabetic nephropathy in particular. (UKPDS 1998, DCCT 1993).

## **1.2 Diabetic nephropathy**

The commonest of the systemic disease involving the kidney is diabetes mellitus. Since the advent of insulin therapy and improve survival of subjects with diabetes nephropathy has proved to be an important consequence of mortality. Diabetic nephropathy plays a significant role as one cause of end stage renal failure in the western world. Diabetic nephropathy is a clinical syndrome characterized by persistent albuminuria, arterial blood pressure elevation, a relentless decline in glomerular filtration rate (GFR), and an associated high risk of cardiovascular morbidity and mortality (Parving *et al*, 2000). This major life-threatening complication develops in approximately 35% of subjects with type 1 diabetes (Rossing *et al*, 1995). The prevalence in type 2 diabetes is higher than type 1; this form of diabetes now contributes to at least 50% (Cooper, 1998) of those with

diabetic nephropathy who develops end stage renal disease (ESRD) and require dialysis or transplantation for survival.

### **1.2.1 Natural history of diabetic nephropathy**

The natural history of diabetic nephropathy has been relatively well defined in type 1 diabetics. In type 2 diabetics, the process remains unclear with regard to the time of onset of disease or the presence of other factors such as hypertension, age or race. However, clinical investigators have been able to classify the development of ESRD based on the onset and duration of the disease. The classifications of ESRD were introduced by Mogensen *et al*, (1993) and divided into five stages as described below.

In stage 1, at the onset of the diabetes, there is glomerular hyperfiltration with hyperperfusion, renal hypertrophy and glomerular capillary hypertension associated with high blood glucose concentrations. At this stage urinary albumin excretion may be normal or slightly elevated. Stage II is a silent phase that follows hyperfiltration and is associated with subtle morphological changes including thickening of the glomerular basement membrane (GBM), glomerular hypertrophy, mesangial expansion, and modest expansion of the tubulointerstitium.

After 7-15 years, with diabetic patients who develop stage III or incipient nephropathy this can be detected clinically by the presence of microalbuminuria. Abnormal urinary albumin excretion cannot be detected by conventional or dipstick methods (semi-



quantitative) but are measurable using sensitive techniques such as quantitative immunoassay. Studies in Type 2 diabetes have suggested that in the transition from normoalbuminuria to microalbuminuria, there is modest rise in blood pressure (systolic/diastolic) of about 3mm/Hg per year.

Stage IV is characterised by the presence of overt nephropathy, with dipstick-positive proteinuria. GFR falls steadily, by about 12ml/min.year, and clinically, measurement of plasma creatinine and microalbuminuria are used to monitor renal function and to indicate the decline of GFR. Hypertension when present in patients at this stage is usually associated with the presence of >500 mg urinary total protein/24 h. However, histological glomerular lesions, found in most long-term diabetics with nephropathy, may include thickened glomerular capillaries, mesangial expansion, intercapillary nodules of glomerulosclerosis of the afferent and efferent arterioles and the presence of glomerular microaneurysms. The mesangial cells of one or more of the glomerular segments produce excessive amounts of a cellular matrix to form spherical nodules known as Kimmelstiel-Wilson nodules. The development of oedema is one of the earliest clinical features of renal impairment, often associated with anaemia and a rather non-specific decline in general health.

Stage V is end stage renal failure, with the presence of severe mesangial expansion, uraemia, hypertension, and serum creatinine concentration of more than 400  $\mu\text{mol/l}$ .

### **1.2.2 Structural, functional, biochemical and physiological aspects of the kidney**

The kidneys play a major role in the homeostatic mechanisms of the human body and reduced renal function strongly correlates with increasing patients morbidity and motility.

The kidneys form a paired organ system, located in the retroperitoneal space, and demonstrate exquisite heterogeneity which consist of three main areas namely the cortex, the medulla and the inner medulla or papilla. The kidneys have both sympathetic and parasympathetic nervous supply whose function appears to be predominantly associated with vasomotor activity. Each kidney receives its blood supply from a single renal artery derived from the abdominal aorta, with the venous return along a renal vein that emerges into the vena cava. The renal artery divides into posterior and anterior elements, which then divide into interlobar, arcuate, interlobular, and ultimately into the afferent arterioles, which expand into the highly specialised capillary bed that form the glomerulus. These capillaries then rejoin to form the afferent arteriole, which then forms the capillary plexuses as well as the elongated vessels such as the vasa recta, which pass around the remaining parts of the nephron, the proximal and distal tubules, the loop of Henle, and collecting duct, providing oxygen and nutrients and removing ions, molecules, and water which are reabsorbed by the nephron.

#### **Nephron**

The functional unit of the kidney is the nephron and each kidney has been reported to contain between 1 and 1.5 million nephrons. There are at least three types of nephrons, which include the superficial, midcortical and juxtamedullary types. Each nephron

consists of a glomerulus and Bowman's capsule, which is next to the proximal tubule (PT) and the following section is the loop of Henle, which leads into the renal medulla. At the junction of the inner and outer medulla, the loop becomes thicker before leading to the distal tubule (DT) and collecting tubule (CT). This section of the nephron then merges with those of other nephrons to form the renal pelvis and ultimately the ureter.

### Glomerulus

The glomerulus consists of a turf of blood capillaries, which is in close contact with Bowmans's capsule. This close alliance enables the ultrafiltration of plasma through three layers of cells which together act as a selective permeable barrier. Glomerular structure and permeability are maintained by the glomerular mesangial cells, thereby altering the glomerular capillary surface area available for filtration. The glomerular basement membrane (GBM) is approximately 300 nm thick in adult humans and consists of three distinct electron-dense layers such as the lamina rara interna, the lamina densa, and the lamina rara externa. The lamina densa consists of mainly type IV collagen embedded in a matrix of glucoprotein and proteoglycans. This forms the main size discriminant barrier to protein passage into the tubular lumen. The other two layers of the GBM are rich in negatively charged polyanionic glucoprotein such as heparin sulphate proteoglycans (HSP). The epithelial cells lining Bowmans's capsule are known as podocytes and have a large number of extensions or foot processes that are embedded in the GBM. The foot processes from adjacent podocytes are interdigitated to form filtration slits, which are covered by highly hydrated anionic mucopolysaccharide that is rich in sialic acid. Consequently, the resulting structure is relatively impermeable to most proteins above 60



kD, but passage of proteins is also modulated by their charge and shape i.e. charge and size selectivity. The final cellular component of the glomerulus are the mesangial cells (MC), which are embedded in an extra cellular matrix (ECM) between the capillaries and play a critical role in the modulation of glomerular blood flow and filtration by contraction and relaxation. MC are of mesenchymal origin and contain contractile elements which express receptors to many different hormones and cytokines, including angiotensin II, insulin-like growth factor 1 (IGF-1), tumour necrosis factor, interleukin-1, transforming growth factor beta (TGF- $\beta$ ) and advanced glycation end products (AGEs). The mesangial matrix, although developmentally and morphologically distinct from the GBM, is composed of essentially the same components, i.e. collagen type IV, laminin and HSP.

### The proximal tubule (PT)

The proximal tubular region of the nephron consists of the proximal convoluted tubule, and a straight segment, also known as the pars recta. Three distinct cell types from the epithelial lining of these proximal structures and the S1, S2 and S3 regions are separated on the basis of their morphological and functional characteristics. The S1 segment, composed of S1-type cells, makes up the beginning and middle portion of the convoluted proximal tubule and are generally columnar with abundant microvilli on the luminal surface, thereby increasing the surface area for reabsorption from tubular fluid by 40 fold. A well developed phagolysosomal system, a large endocytotic apparatus of numerous apical vacuoles and numerous mitochondria are also present in this segment. The S2 segment forms the remaining portion of the proximal convoluted tubule and the initial

portion of the straight segment. S2 cells have fewer microvilli and mitochondria than S1 cells and they also contain the majority of the PT lysosomes. Finally, the remaining portion of the pars recta is the S3 segment which consists of the end of the straight portion and the beginning of the thin descending loop Henle. Cells in this region have few microvilli and mitochondria. The S1 and S2 segments perform the majority of the solute and fluid reabsorption coupled largely to sodium reabsorption. Large basolateral membrane (BLM) interdigitations are found within the PT providing a large surface area for  $\text{Na}^+/\text{K}^+$  transport, which maintains the osmotic gradients necessary for fluid reabsorption within the PT.

### Loop of Henle and distal tubule (DT)

The loop of Henle is divided into the descending and ascending limbs. These limbs form a hairpin loop, which either extend deeply into (juxtramedullary) or reach only just into the medulla (cortical). The terminal segment of the distal tubule is the convoluted part. Each segment of the loop of Henle and the subsequent distal section have different permeability properties, allowing the production of concentrated urine, as well as controlling intra and extracellular osmolality and pH. Part of the distal tubule is specially close to the glomerulus and the afferent and the efferent arterioles. Specialised cells, called the macula densa, which are in this area respond to the sodium and chloride composition of tubular fluid in order to maintain water and electrolyte homeostasis (i.e. tubuloglomerulo feed back).



### Filtration, reabsorption and secretion

Glomerular filtration is a passive process dependent on the pressure within the glomerular capillary network which in turn depends on the surface area and intrinsic permeability of the glomerulus. The hydrostatic and oncotic pressure gradients across the capillary walls are determined by both flow and resistance. In the normally functioning kidney, molecules of  $\geq 50 \text{ \AA}$  are not filtered due to size, shape or charge, whilst molecules  $\leq 40 \text{ \AA}$  are freely permeable. These include glucose, amino acids, urea,  $\text{Na}^+$  and  $\text{K}^+$  ions. The filtrate entering the nephron is isotonic with, and of similar composition to, plasma except for the presence of large molecular weight proteins, which remain within the plasma. Approximately 60-70% of the filtrate volume (water, sodium, and urea) is reabsorbed in the PT whilst some ions and molecules are actively secreted further down the nephron to maintain blood ionic concentration and volume. The sodium concentration in the PT cell effects levels of energy generation and  $\text{Na}^+$  ions enter passively down an electrical gradient (-70mV) and are then actively transported across the BLM via  $\text{Na}^+-\text{K}^+$ -ATPase and  $\text{Na}^+-\text{H}^+$ -ATPase activity which allows the reabsorption and secretion of other solutes such as  $\text{Cl}^-$ ,  $\text{K}^+$ ,  $\text{H}^+$  ions, to balance the  $\text{Na}^+$  ion movement.

### Renal blood flow (RBF) and glomerular filtration rate (GFR)

According to the myogenic theory, increased tension of the afferent arterioles, brought about by an increase in perfusion pressure, causes automatic contraction of the smooth muscle fibres in the vessel wall, thereby increasing the resistance to flow and so keeping

the flow constant in spite of the increase in perfusion pressure. Tubulo-glomerular feedback (TGF) mechanisms are also involved in supporting this process. TGF is a mechanism in which changes in DT fluid composition are detected by the macula densa and, as a consequence, alter the vascular elements of the glomeruli, thereby affecting single nephron GFR, RBF and GFR are also under hormonal and nervous control. An intact renin-angiotensin-aldosterone system (RAAS) is required for the normal regulation of GFR.

Measurement of GFR

GFR is considered to be the most reliable measure of the glomerular functional capacity of the kidneys and is often thought of as indicative of the number of functioning nephrons. An estimate of the GFR can be made by measuring the urinary excretion of a substance which is completely filtered from the blood by the glomeruli and which is not secreted, reabsorbed or metabolised by the renal tubules. Experimentally, inulin has been found to meet these requirements. However, measurement of creatinine clearance is commonly used method for estimation of the GFR in the routine clinical laboratory.

$$\text{Clearance} = \frac{U \times V \text{ ml/min}}{P}$$

U = urinary creatinine concentration (μmol/l),

V = urine flow rate [ml/min or (1/24 h)/1.44m<sup>2</sup>]

P = plasma creatinine concentration (μmol/l)

Furthermore, the GFR can also be estimated using one of several algorithms. The best known algorithm is the Cockcroft and Gault formula (Cockcroft & Gault, 1976) as given below.

$$\text{CrCl (mL/min/1.73 m}^2\text{)} = \frac{(140\text{-age in years}) \times 2.12 \times \text{weight (kg)} \times k}{\text{serum creatinine} \times \text{BSA (m}^2\text{)}}$$

BSA = body surface area, K = 0.85 for women and 1.00 for men

### 1.2.3 Structural and functional changes in diabetic kidney

Nephromegaly (renal enlargement) was first described in diabetes more than a century ago (Ritz *et al*, 1989) and is an early feature of both experimental and human diabetes. In animals, nephromegaly occurs within four days of diabetes onset and most type 1 diabetics have large kidneys at diagnosis. Consequently, this enlargement is mostly due to a combination of tubular hypertrophy and hyperplasia and interstitial expansion, and is probably a response to increased glucose and fluid filtration and their active reabsorption. Furthermore, glomeruli only account for 1% of total kidney volume, so their contribution to whole organ enlargement is significant (Bilous, 2001).

Glomerular enlargement has been a recognised feature of diabetes, and is present both in early and later stages of the disease (DeFronzo, 1995). Early glomerular enlargement in experimental diabetes is the result of an increase in capillary filtration surface area caused by an increase in capillary length, or number, or both. In experimental diabetes, early glomerular enlargement coincides with increased glomerular filtration (Mauer *et al*, 1975). This so-called hyperfiltration has been proposed as an important pathophysiological factor for diabetic glomerulopathy (Mauer *et al*, 1984). Glomerular enlargement has therefore been considered as a factor predisposing the subject to progression of glomerulopathy, perhaps by an increase in capillary wall radius in response to raised intraglomerular pressure, thus leading to increased capillary wall tension (Bilous, 2001). Furthermore, the end result of diabetic glomerulopathy is a globally sclerosed, non-functioning glomerulus. Such glomeruli can appear either as relatively acellular, eosinophilic globes, which ultimately hyalinize and become



reabsorbed or as collapsed structures with an irregular crenated basement membrane. The former appearance is consistent with an internal glomerular obliteration by mesangial expansion, the latter more with ischaemia. These interpretations are supported by reported positive correlations of percentage of globally sclerosed glomeruli with mesangial expansion and afferent arteriolar hyalinosis (WHO, 1985).

GBM thickening can be demonstrated in most diabetic subjects, irrespective of the severity of their nephropathy, although those with heavier proteinuria tend to have thicker membranes. It is thus not as specific a sign of renal complication as the diffuse mesangial lesion, although those subjects with nephropathy tend to have thicker GBMs than age and diabetes duration matched subjects with 'normal' renal function (Bilous *et al*, 2001). Most of the increase in matrix is due to type IV collagen accumulation. There is, however, a net loss of proteoglycan, which is also dispersed throughout the thicker membranes. This loss is probably to result in a loss of negative electrostatic charge and thus permit the passage of positively charged proteins such as albumin (Bilous, 2001).

The Kimmelstiel-Wilson nodule is more specific for diabetic nephropathy but occurs in only 20-70% of diabetics who demonstrate the diffuse lesion. Furthermore, there are no obvious changes in endothelial or epithelial cells in glomerulopathy, except that there is a widening of podocyte foot processes. This development may be a ubiquitous response to increased protein passage across the GBM, as its observed in other proteinuric states (Bilous, 2001).



A tubulo-interstitium compartment is also a major feature of diabetic nephropathy and an important predictor of renal dysfunction. Renal enlargement in diabetic animals is due to an initial tubular cell hyperplasia and subsequently to cell hypertrophy. These changes are associated with alteration in expression of growth factors such as TGF- $\beta$  and can be prevented by appropriate insulin therapy. Tubular basement membrane (TBM) probably has a similar composition and structure to GBM but is almost twice as wide. However, the TBM structure has still not been widely studied. Diabetics develop TBM thickening about two to three times the value observed in their non-diabetic siblings (Steffes *et al*, 1985). Furthermore, macromolecular penetration of the interstitial space may activate fibrosis and would be further facilitated by disruption of the TBM. AGEs also have been demonstrated to increase pore size in bovine TBM, which may also result in increased protein permeation (Anderson *et al*, 1993).

#### **1.2.4 Clinical and biochemical aspects of diabetic nephropathy**

##### **1.2.4.1 Definition of nephropathy**

Clinical diabetic nephropathy is defined as the development of persistent proteinuria and hypertension, and it is preceded by incipient diabetic nephropathy, characterised by persistent microalbuminuria. Once clinical diabetic nephropathy has developed, progression is difficult to prevent by improved metabolic control or through the use of antihypertensive therapy. (Andersen *et al*, 1983; Mogensen and Christensen, 1984; Okada *et al*, 1996).

#### **1.2.4.2 Diagnosis of diabetic nephropathy**

The diagnosis of diabetic nephropathy is only definitively made by renal biopsy, but this is rarely necessary clinically where the diagnosis is based on both the clinical and biochemical abnormalities demonstrated the kidney, such as the presence of proteinuria, development of a progressive rise in blood pressure, and a progressive and relentless decline in renal function towards end stage renal failure. Elevations of urinary albumin excretion are used to define both the diagnosis of diabetic nephropathy and its progression. An increase in albumin excretion is taken as the hallmark of diabetic nephropathy (Viberti *et al*, 1982; Mogensen, 1984).

#### **1.2.4.3 Microalbuminuria**

##### **Clinical significance**

Microalbuminuria is one of the earliest signs of renal insult in diabetes and is currently the main focus of attention, as it is also associated with increased risk of morbidity and premature death from cardiovascular disease (Haffner *et al*, 1990). The presence of microproteinuria in general, or microalbuminuria in particular, reflects loss of charge selectivity and an increase in capillary permeability in the kidneys and other organs. Microalbuminuria is a predictor of chronic renal failure (overt nephropathy) in diabetes mellitus; its presence here is termed incipient nephropathy.

##### **Definition**

Microalbuminuria, defined as an increased urinary albumin excretion (UAE) detectable only by sensitive immunoassay (Winocour, 1992) expressed either by time or with

reference to creatinine concentration), has been used for many years as a predictor of incipient nephropathy in diabetics (Viberti *et al*, 1982; Mogensen, 1984). The ‘gold standard’ of microalbuminuria measurement is based on the excretion rate of albumin in a timed urine collection, while for more rapid estimations the urinary albumin concentration in an early morning mid stream specimen of urine may be used. In order to correct for variations in body fluid balance, the latter is normally referenced against the urinary creatinine concentration as the albumin:creatinine ratio (ACR) values.

**Table: 1.2.4.3 Classification of albuminuria**

	Normalbuminuria	Microalbuminuria	Macroalbuminuria
Albumin excretion rate (mg/24h)	< 30	30-300	> 300
Albumin excretion rate (µg/min)	< 20	20-200	> 200
Albumin concentration (mg/L)	< 20	20-200	> 200
ACR(mg/g creatinine)	< 30	30-300	> 300
ACR (mg/mmol creatinine)	< 3.5	3.5-35	> 35

Adopted from Bilous, 2001

Progression

Microalbuminuria may initially be transient in nature, but may become persistent and result in the patient progressing to end stage renal failure if left untreated. Indeed, microalbuminuria may progress to ESRD in 7-10 years after onset of diabetes (American



Diabetic Association/ADA, 1997). In subjects with type 1 diabetes, about 80% of whom develop persistent microalbuminuria if left untreated, develop overt nephropathy within 10-15 years, accompanied by hypertension. This may eventually lead to end stage renal renal failure within a further 10-20 years without appropriate therapeutic intervention (ADA, 1997). The higher proportion of individuals with type 2 diabetes who develop microalbuminuria and overt nephropathy shortly after the diagnosis of diabetes is probably because diabetes has been present for many years before the diagnosis was made. However, overall 20-40% of type 2 diabetics with microalbuminuria progress to overt nephropathy which ultimately may lead to ESRD (ADA, 2002).

### Pathophysiology

The intimate relationship between low-level albumin excretion and vascular permeability makes UAE highly sensitivity to the presence of any inflammatory process, including cardiovascular disease (Mogensen, 1976; Haffner *et al*, 1990). Almost all filtered albumin is reabsorbed by the proximal tubule via a high-affinity, low-capacity endocytotic mechanism (Hartland and Gosling, 1999). Since tubular mechanisms for albumin reabsorption are near saturation, urinary albumin excretion would increase following any increase in tubular load. Glomerular permeability to albumin is dependent on endothelial charge selectivity as well as size selectivity. The negative charge conferred on the glomerular membrane by its constituent glycoproteins plays a role in restricting the permeability of anionic proteins. Loss of glomerular charge selectivity has been found in both diabetic and non-diabetic subjects with microalbuminuria. However, the mechanisms underlying microalbuminuria still remain to be fully elucidated (Hartland and Gosling, 1999).



### Screening

Annual screening for microalbuminuria will allow the identification of those diabetics with either nephropathy or at risk of developing nephropathy. A routine urinalysis should be performed at diagnosis in subjects with type 2 diabetes. Conversely, microalbuminuria rarely occurs with short duration of type 1 diabetes or before puberty and therefore, screening should begin with puberty and after 5 years disease duration. If the urinalysis is positive for protein, a quantitative measure is frequently helpful in the development of a treatment regime. Screening for microalbuminuria can be performed by three methods: 1) measurement of the albumin to creatinine ratio in a random spot urine collection; 2) 24h collection with creatinine, allowing the simultaneous measurement of creatinine clearance; and 3) timed (e.g. 4h or overnight) collection (ADA, 1997).

### **1.2.5 Treatment of diabetic nephropathy**

#### Effective of glycemic control

The DCCT, the UKPDS, the Stockholm and Intervention Study have demonstrated definitively that intensive diabetes therapy can significantly reduce the risk of the development of microalbuminuria and overt nephropathy in subjects with diabetes. However, an improvement in renal function, which is already restricted, can be attained by limiting maintaining blood glucose concentrations close to reference values (mean HbA<sub>1c</sub> 6.5%) and intensified blood pressure treatment.

#### Control of blood pressure and antihypertensive agent

Both systolic and diastolic hypertension significantly accelerate the progression of diabetic nephropathy, and aggressive antihypertensive management is able to greatly

decrease the rate of decline of GFR. According to guidelines in the sixth report of the Joint National Committee (JNC) on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, blood pressure levels above 135/85 mm Hg in a diabetic is abnormal. Treatment should be directed at lowering the systolic level to about 100 to 110 mm Hg (JNC, 1997). The positive response to antihypertensive treatment coupled with the concept that often there is a progressive deterioration of renal function regardless of the underlying aetiology gave rise to the idea that haemodynamic factors may be critical in furthering the depletion in GFR. In this hypothesis, damage to glomeruli causes changes in the microcirculation that result in hyperfiltration occurring in the remaining glomeruli with increased intraglomerular pressure and increased sensitivity to angiotension II; the single nephron hyperfiltration with intraglomerular hypertension is in itself damaging (ADA, 1997). Initially, Angiotensin Converting Enzyme (ACE) inhibitors can reduce the degree of microalbuminuria and can reduce the rate of progression of diabetic nephropathy to a greater degree in both type 1 and type 2 diabetics (Mogensen, 1976; DeFronzo, 1995; ADA, 1997).

### Protein Restriction

Dietary protein restriction has been demonstrated to decrease albuminuria and reduce the rate of deterioration glomerular function in various renal diseases. Both animal and human studies have demonstrated that restriction of dietary protein intake also reduces diabetic intraglomerular pressure and retards the progression of renal disease and diabetic nephropathy. The consensus is to prescribe a protein intake of approximately the adult recommended dietary allowance (RDA) of  $0.8 \text{ g kg}^{-1} \text{ day}^{-1}$  (approximately 10% of daily calories) in the subjects with overt nephropathy. However, it has been suggested that

once the GFR begins to decline, further restriction to  $0.6 \text{ g kg}^{-1} \text{ day}^{-1}$  may prove useful in slowing any further decline of GFR. (ADA, 1997).

#### Renal replacement therapy (RRT)

RRT consists of haemodialysis, continuous ambulatory peritoneal dialysis (CAPD) and renal transplantation. Renal transplantation is the treatment of choice for those under 60 years of age and should be considered when the serum creatinine concentration reaches about 500-800  $\mu\text{mol/l}$  (Bilous, 2001).

#### **1.2.6 Pathogenesis of diabetic nephropathy**

As described previously, diabetic nephropathy is a major cause of renal failure. Several factors have been postulated in the pathogenesis of diabetic nephropathy including association of genetic factors, haemodynamic factors, vasocoactive factors, growth factors, renal structural factors, and hyperlipidaemia. However, it has been confirmed that chronic hyperglycaemia plays a significant role, and it is the major culprit in the development of diabetic nephropathy. Consequently, the present study focuses on several risk factors associated with abnormal glucose metabolism in general and the association between free radicals and oxidative stress in the pathogenesis of diabetic nephropathy in particular as described in Section 1.3. below.



## 1.3 Free Radicals

### 1.3.1 Basic concept of free radicals

In 1956, Denham Harman, father of the free radical theory postulated that free radicals produced during aerobic respiration cause cumulative oxidative damage, resulting in aging and death. Free radicals are generally considered harmful byproducts of oxidative metabolism (Oberley, 1988), causing molecular damage in living systems. This concept has implications in numerous biological phenomena such as cellular aging, mutagenesis, inflammation, and other pathologies. Furthermore, it has been suggested that free radicals are implicated in the process in part for the development of diabetic microangiopathy and macroangiopathy (Barnett, 1991), and excessive free radical production has been reported in diabetics with chronic renal failure treated by haemodialysis (Loughrey *et al*, 1994). Consequently, free radical mechanisms have been implicated in the pathogenesis of tissue damage in diabetes (Oberley, 1988; Packer, 1993; Wolff, 1993; Halliwell and Gutteridge, 1998).

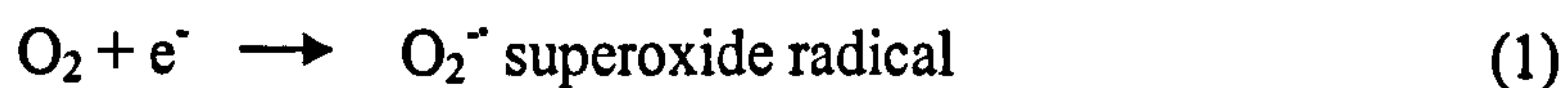
The term “free radical” can be defined as any atoms or molecules that contain an unpaired electron in its outer orbit that can exist independently (Halliwell, 1994; Halliwell and Gutteridge, 1998). As a result, they can be highly reactive, although this varies from radical to radical, reacting locally to accept or donate electrons to other molecules to achieve a more stable state. Ground state  $O_2$  ( $^3O_2$ ) has two unpaired electrons each located in a different antibonding orbital. An oxidizing agent, such as  $O_2$  is effective at absorbing electrons from the molecule it oxidizes (Halliwell and Gutteridge, 1998). The



collective terms reactive oxygen species (ROS) or active oxygen species have been applied for a variety of free radicals and non-radicals intermediates (Halliwell, 1994).

### 1.3.2 Pathophysiology of free radicals

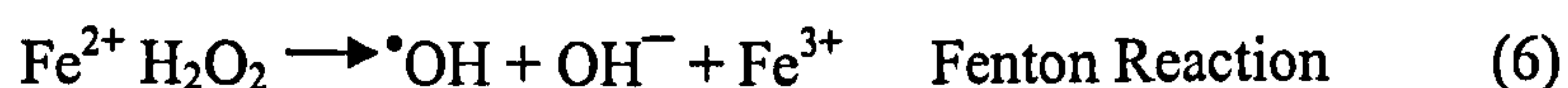
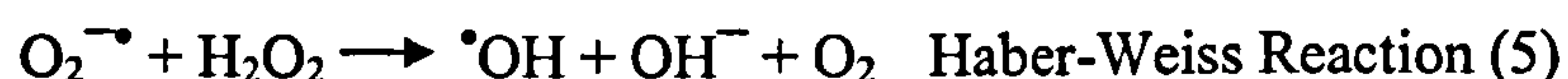
Free radicals are formed in large amounts as an unavoidable by product of many biochemical processes and in some instances, deliberately, such as in activated neutrophils. In addition, free radicals can be generated in the body in response to electromagnetic radiation from the environment and acquired directly as oxidizing pollutants such as ozone and nitrogen dioxide (Halliwell, 1994). However, most of the free radicals in biological systems are oxygen-derived free radicals. The complete reduction of oxygen to H<sub>2</sub>O requires four steps and the generation of several free radicals and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which is in itself, not a free radical. H<sub>2</sub>O<sub>2</sub> is however, considered a reactive oxygen species (ROS) because of its ability to generate highly reactive hydroxyl free radicals through interactions that reactive transition metals (Gutteridge, 1995; Clarkson and Thompson, 2000). The complete reduction of oxygen can be explained in the following equations.



Each of these oxygen-derived intermediates are considered highly reactive species because their electron configurations allow for the attraction of electrons from other molecules, resulting in the formation of other free radicals that are capable of reacting with yet another molecule. This chain reaction is thought to contribute to lipid peroxidation, DNA damage, and protein degradation during oxidatively stressful events. Although all the intermediates are potentially reactive, the intermediates vary in their biological importance. The superoxide radical is the leading oxygen-derived free radical and, unlike the other oxygen derived intermediates, can lead to the formation of additional ROS (Harris, 1992). Furthermore, the protonation of superoxide anion radicals results in the formation of perhydroxyl radical ( $\text{HO}_2^\cdot$ ), a much more aggressive radical than the superoxide radical itself. In addition, superoxide acts as a Bronsted base in aqueous solutions to shift the acid-base equilibrium to form a hydroxyl ( $\cdot\text{OH}$ ) radical thereby forming hydrogen peroxide in acidic environments. (Halliwell, 1994; Gutteridge, 1995; Clarkson and Thompson, 2000). Hydrogen peroxide, although not a free radical by definition, is a biologically important oxidant because of its ability to generate the hydroxyl radical, an extremely potent radical species. Furthermore, because of its non-ionized and low charge state, hydrogen peroxide is able to diffuse through hydrophobic membranes, as observed with the leakage of hydrogen peroxide from mitochondria.

Hydroxyl radicals are formed not only by the reduction of hydrogen peroxide but also through the interaction between hydrogen peroxide and the reduced forms of metal ions, i.e., copper and iron. The ability of the hydroxyl radical to remove or add hydrogen molecules to the unsaturated hydrogen bonds of organic lipids makes it potentially one of the most reactive oxidants in biological systems (Clarkson and Thompson, 2000).

Furthermore, the hydroxyl radical is the most powerful oxidant that can readily attack polyunsaturated fatty acids to initiate lipid peroxidation (Gutteridge, 1995). Consequently, the most toxic oxygen free radical involved in pathological processes is the hydroxyl radical ( $\cdot\text{OH}$ ). In biological systems,  $\cdot\text{OH}$  derives from the less toxic superoxide anion radical ( $\text{O}_2^{\cdot-}$ ) and  $\text{H}_2\text{O}_2$  via the Haber-Weiss and Fenton reactions (Gutteridge, 1995).



### 1.3.3 Antioxidant defence systems

Antioxidants are defined as any substance that when present at low concentrations, compared with those of the oxidative substrate considerably delays or inhibits oxidation of the substrate (Halliwell, 1994; Gutteridge, 1995; Halliwell and Gutteridge, 1998). Antioxidants can act at many different stages in an oxidative sequence including removing oxygen or decreasing local oxygen concentrations, removing catalytic metal ions, removing key ROS such as oxygen and hydrogen peroxide, scavenging initiating free radicals, breaking the chain of an initiated sequence, and quenching or scavenging singlet oxygen species (Gutteridge, 1995; Halliwell and Gutteridge, 1998). Furthermore, a variety of antioxidant defense systems operates, including enzymatic and non-enzymatic antioxidants (Halliwell, 1994). Enzymatic antioxidants directly involved in the detoxification of ROS are super oxide dismutase (SOD) and hydroxyperoxidases such as



catalase (CAT) and glutathione peroxidase (GSHPx) a selenium-containing enzyme glutathione (GSH) (Gutteridge, 1995; Halliwell, 1994).

Cells have formidable defence mechanisms against oxidative damage of which some may not be readily recognisable as antioxidants (Gutteridge, 1995). Enzyme such as SOD rapidly promote the dismutation of superoxide into hydrogen peroxide and oxygen at a rate considerably faster than it occurs uncatalyzed. Two different superoxide dismutases are found in mammalian tissue, namely a Cu/Zn-containing enzyme which is found in the cytoplasm of most cells, and a further Mn-containing enzyme present within the mitochondrial compartment (Rice-Evans, 1994; Gutteridge, 1995). Both enzymes catalase the same reaction as shown below.



Hydrogen peroxide, a product of the dismutation reaction, can be destroyed by two enzymes, catalase and glutathione peroxidase. Glutathione peroxidase can metabolise hydrogen peroxide, generated by SOD, by oxidizing the tripeptide glutathione into its oxidized form (GSSG). In addition, catalase transforms hydrogen peroxide into water and oxygen (Gutteridge, 1995) as shown below.



Apart from these endogenous antioxidants, an important source of antioxidants is in the diet, which contains numerous compounds exhibiting antioxidant activity. The most



prominent dietary antioxidants are tocopherols, the fat-soluble vitamin (vitamin E), ascorbate water-soluble vitamin (vitamin C) and carotenoids. Furthermore, other antioxidants such as albumin and other proteins including ceruloplasmin and transferrin also protect against oxidative injury by binding the transition metals  $\text{Fe}^{+2}$  and  $\text{Cu}^{+2}$  thereby preventing generation of the hydroxyl radical via the Fenton reaction (Gutteridge, 1995).

#### 1.3.4 Oxidative stress

As previously described, oxygen-derived free radicals are constantly formed in the body during normal metabolic processes. When free radical formation is greatly increased, or protective antioxidant mechanisms compromised, a state of oxidative stress will result. If oxidative stress persists, it will eventually lead to molecular damage and tissue injury (Symons and Gutteridge, 1998). Consequently, oxidative stress has been defined as a disturbance in the balance between the production of free radicals (ROS) and antioxidant defenses, which may lead to tissue injury (Halliwell, 1994). Subjects with diabetes may be especially prone to oxidative stress, which enhances the development and progression of diabetic micro and macrovascular complications (Baynes, 1991; West, 2000). Animal and human studies and *in vitro* experiments all suggest a role of oxidative stress, via an increased formation of free radicals in the pathophysiology of diabetic microvascular complications (Baynes, 1991; West, 2000) such as nephropathy and retinopathy.

### **1.3.5 Free radical-mediated oxidative tissue damage**

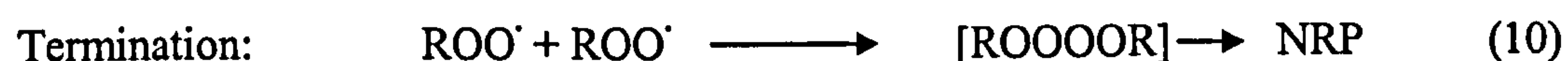
The human body has a multiplicity of different antioxidant defense mechanisms (Halliwell and Chirico, 1993). If the defensive processes are overwhelmed, free radicals can then become highly destructive to cells and tissues. During oxidative stress, the prooxidant-antioxidant balance is tipped in favour of the former, and this may be due to exogenous sources of free radicals or other endogenous stresses (Rice-Evans 1994). However, oxidative stress can produce major interrelated derangements of cell metabolism, including DNA damage, protein damage and peroxidation of lipids (Halliwell and Chirico 1993). The relative importance of damage to different molecule as targets in producing cell injury or death by improving oxidative stress depends on duration, degree of stress underlying mechanism and the nature of the system stressed (Halliwell and Chirico, 1993).

#### **1.3.5.1 Lipid peroxidation**

The free radical oxidation of polyunsaturated fatty acid (PUFA) in biological systems is known as lipid peroxidation (Gutteridge, 1995). A PUFA contains two or more double bonds, and the presence of an increasing number of double bonds in fatty acids makes it more susceptible to oxidative damage by free radicals and peroxidation (Symons and Gutteridge, 1998). Both monounsaturated and saturated fatty acids are much less reactive and do not usually participate in lipid peroxidation (Abuja and Albertini, 2001).

The present study focuses on non-enzymatic lipid peroxidation or lipid oxidation reactions. In the non-enzymatic lipid peroxidation process, the addition of oxygen yields

a lipid peroxy radical, which is considered a hallmark of peroxidising lipids. Lipid peroxidation consists of mainly three processes, namely initiation, propagation and termination (reactions 7-10).



Lipid peroxidation can be initiated by any primary free radicals ( $^\cdot\text{OH}$ ,  $\text{O}_2^{\cdot-}$ ) of sufficient reactivity to substitute an allylic hydrogen atom from a reactive methylene group of PUFA side-chains. In the initiation step, polyunsaturated lipids (RH) may form alkyl radicals ( $\text{R}^\cdot$ ) which react very rapidly with oxygen to form peroxy radicals ( $\text{ROO}^\cdot$ ). In the propagation step, a chain reaction with more lipids produces hydroperoxides ( $\text{ROOH}$ ), i.e. primary oxidation products. Propagation reactions can repeat themselves many times. Thus, an initial event triggering lipid peroxidation can be amplified with the availability of oxygen and PUFA side chains (Rice-Evans, 1994). Consequently, the accumulation of hydroperoxides and their subsequent decomposition to alkoxy and peroxy radicals can accelerate the chain reaction with PUFA leading to oxidative damage in cells, membranes and lipoproteins (Rice-Evans, 1994). Under such conditions where lipid peroxidation is continuously initiated, a termination reaction limits the extent of lipid peroxidation,



yielding non-radical products (NRP), and destroying two radicals at the same time (Abuja and Albertini 2001).

In contrast, the potential consequences of the peroxidation of membrane lipids include loss of polyunsaturated fatty acids; loss of decreased lipid fluidity, altered membrane permeability, effects on membrane-associated enzymes, altered iron transport, release of material from subcellular compartments and the generation of cytotoxic metabolites of lipid hydroperoxide (Rice-Evans, 1994). Furthermore, cleavage of the carbon bonds during lipid peroxidation reactions results in the formation of aldehyde products such as cytotoxic alkanals and alkenals, as well as alkanes. The breakdown products of lipid peroxidation, for example alkanals such as malondialdehyde (MDA), and hydroxyl alkenals such as 4-hydroxynonenal (HNE), have all demonstrated cytotoxic properties (Rice-Evans, 1994).

#### **1.3.5.2 Mechanism of antioxidant actions on lipid peroxidation**

In order to prevent overload of free radicals and peroxides, biological systems possess sophisticated antioxidant defensive mechanisms, which operate both in the intra and extracellular aqueous phases, and also in membranes (Rice-Evans, 1994). In extracellular fluids, numerous antioxidants are present; they can either prevent initiation or intercept lipid peroxy radicals involved in the propagation phase. In human plasma there are abundant binding proteins present in order to prevent metal-induced catalysis. In addition, cell membranes and lipoproteins contain lipophilic antioxidants, which are able



to react with lipid peroxyl radicals, eventually terminating the chain reaction (Abuja and Albertini, 2001).



The tocopherol (Toc) radical (TocO<sup>·</sup>), located in lipid membranes, can be reduced to TocOH by ascorbate, located in the aqueous phase. It is probable that physiological homeostasis requires a balance presence of antioxidants located in both aqueous and lipid phase. There are also synergistic interactions between the tripeptide glutathione (GSH) and vitamin E (α- tocopherol), which may involve membrane-bound enzymes. It has been demonstrated that under specific experimental conditions *in vitro*, in the absence of water-soluble antioxidants, TocO<sup>·</sup> can abstract H<sup>·</sup> from an adjacent fatty acid and therefore act as a prooxidant. Consequently, removal of lipid hydroperoxides is an essential mechanism for preventing such re-formation of free radicals. (Rice-Evans, 1994; Abuja and Albertini, 2001).

### **1.3.6 The role of free radicals and oxidative stress in the pathogenesis of diabetes**

There is emerging evidence suggesting that subjects with diabetes have concomitant increased free radicals production and depletion of cellular antioxidant defense systems. It is well established that alloxan and streptozotocin induced diabetic animals become hyperglycaemic as the result of destruction of  $\beta$ -cells of the pancreas by free radicals (Oberley, 1988). It is probable that in certain genotypes, glycation and glycoxidation lead to an increased susceptibility to oxidative stress than in other genotypes. This would be the genotypes in which  $\beta$ -cell destruction leads to the development of type 2 diabetes (West, 2000). Pancreatic  $\beta$ -cell are especially vulnerable to oxidative stress, probably because of their low free radical scavenging enzyme capacity reflected in low SOD, catalase and glutathione peroxidase activities. Recent studies have reported a direct link between the imbalance of oxidative stress and antioxidants leading to impaired glucose uptake. In adiposities, glucose uptake was rapidly decreased when they were incubated with glucose oxidase which resulted in a steady production of hydrogen peroxide. The reduction of insulin-dependent 2-deoxyglucose uptake was consequently accompanied by decreased  $PI_3$  kinase activity and GLUT4 translocation. These observations support the suggestion that free radicals and antioxidant depletion could impair insulin-mediated  $PI_3$  kinase activity, which results in impaired GLUT4 translocation and defective insulin-mediated glucose uptake. Increased oxidative stress, in addition to antioxidant depletion, leads to decreased glucose uptake were also observed in muscle cells. Furthermore, depletion of antioxidants accompanied by decreased glucose uptake has also been observed in subjects with type 2 diabetes (Rosen *et al*, 2001). These observations lead to

the hypothesis that the imbalance of free radicals and antioxidants is an important pathogenic factor affecting insulin-signalling pathways (Ceriello, 2000; Rosen *et al*, 2001). However, clinical and experimental studies have demonstrated that supplementation with antioxidants such as vitamin E and  $\alpha$ -lipoic acid stimulate glucose uptake through activation of the insulin-signalling pathway and provide protective effects to diabetic state (Rosen *et al*, 2001).

### **1.3.7 Biochemical pathways of oxidative stress in diabetic complications**

Although the underlying patho-mechanisms remain incompletely understood, it can be postulated that oxidative stress due to chronic hyperglycaemia may play a significant role in the pathogenesis of diabetic nephropathy, retinopathy and neuropathy. Several biochemical pathways have emerged as being predominant potential pathophysiological mechanisms of oxidative stress that can be associated with hyperglycaemia (Baynes, 1991) in diabetes mellitus. Furthermore, diabetes associated oxidative stress is probably a result of both an increased production of plasma free radical concentrations and a significant reduction in antioxidant defense mechanisms (Mercuri *et al*, 2000).

#### **1.3.7.1 Advanced glycation end products (AGEs) pathway.**

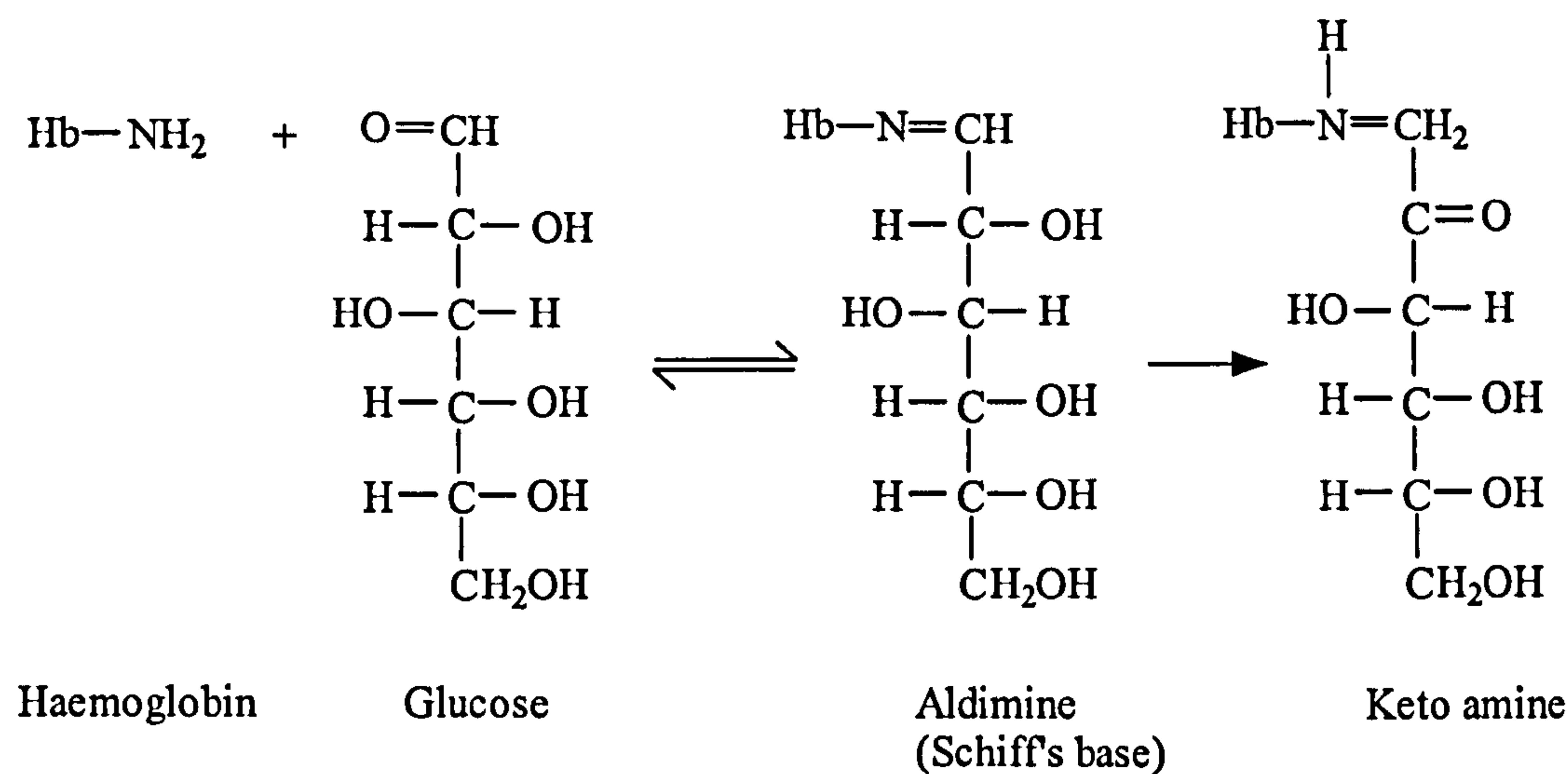
Reducing sugars such as glucose react non-enzymatically with amino groups in proteins and initiate glycation, the early stage of the Maillard reaction. This process begins with the conversion of a reversible Schiff base Amadori adducts. The occurrence of this class of non-enzymatic glycosylated adducts *in vivo* was established after chemical structural analysis of the minor haemoglobin species, HbA<sub>1c</sub> (Bucala and Cerami, 1992). HbA<sub>1c</sub> is



formed in the erythrocytes by a non-enzymatic reaction between glucose and haemoglobin. The aldehyde group of the glucose and a free amino group of the haemoglobin first react to form a Schiff's base (aldimine, unstable form). Subsequently a stable ketoamine is formed in a reaction known as Amadori rearrangement (Bucala and Cerami, 1992) (Figure 1.3.7.1). The increase in the ambient circulating levels of HbA<sub>1c</sub> in diabetes is due to a shift in the equilibrium between the serum glucose concentration and the amount of protein-bound Amadori products. Since haemoglobin has a half-life of sixty days and Amadori product formation reaches equilibrium over 28 days, significant elevations of HbA<sub>1c</sub> can be measured during periods of prolonged hyperglycaemia (Koenig *et al*, 1976). Furthermore, in the intermediate stage of the Maillard reaction, the Amadori products can then undergo further rearrangement, oxidation, multiple dehydration and polymerization resulting in the formation of advanced glycation end products (AGEs) (Bucala and Cerami, 1992). AGEs are irreversibly formed, and their accumulation has been demonstrated with aging, atherosclerosis, and diabetes, especially associated with long-lived proteins such as collagens, lens crystallins, and nerve proteins (Bucala and Cerami, 1992; Bucala *et al*, 1993). It has been suggested that the formation of AGEs not only modifies protein properties, but also induces biological damage *in vivo* (Yim *et al*, 1995). AGEs deposited in the arterial wall, could themselves generate free radicals capable of oxidizing vascular wall lipids and accelerate atherogenesis in hyperglycaemic diabetics (Yim *et al*, 1995, Esterbauer *et al*, 1996). The molecular structures of some AGEs have been identified as N<sup>ε</sup>-carboxymethyllysine, pentosidines and pyrraline. In the presence of molecular oxygen, the formation of these products from sugars is catalyzed by transition metal ions via glycoxidation, which oxidizes Amadori



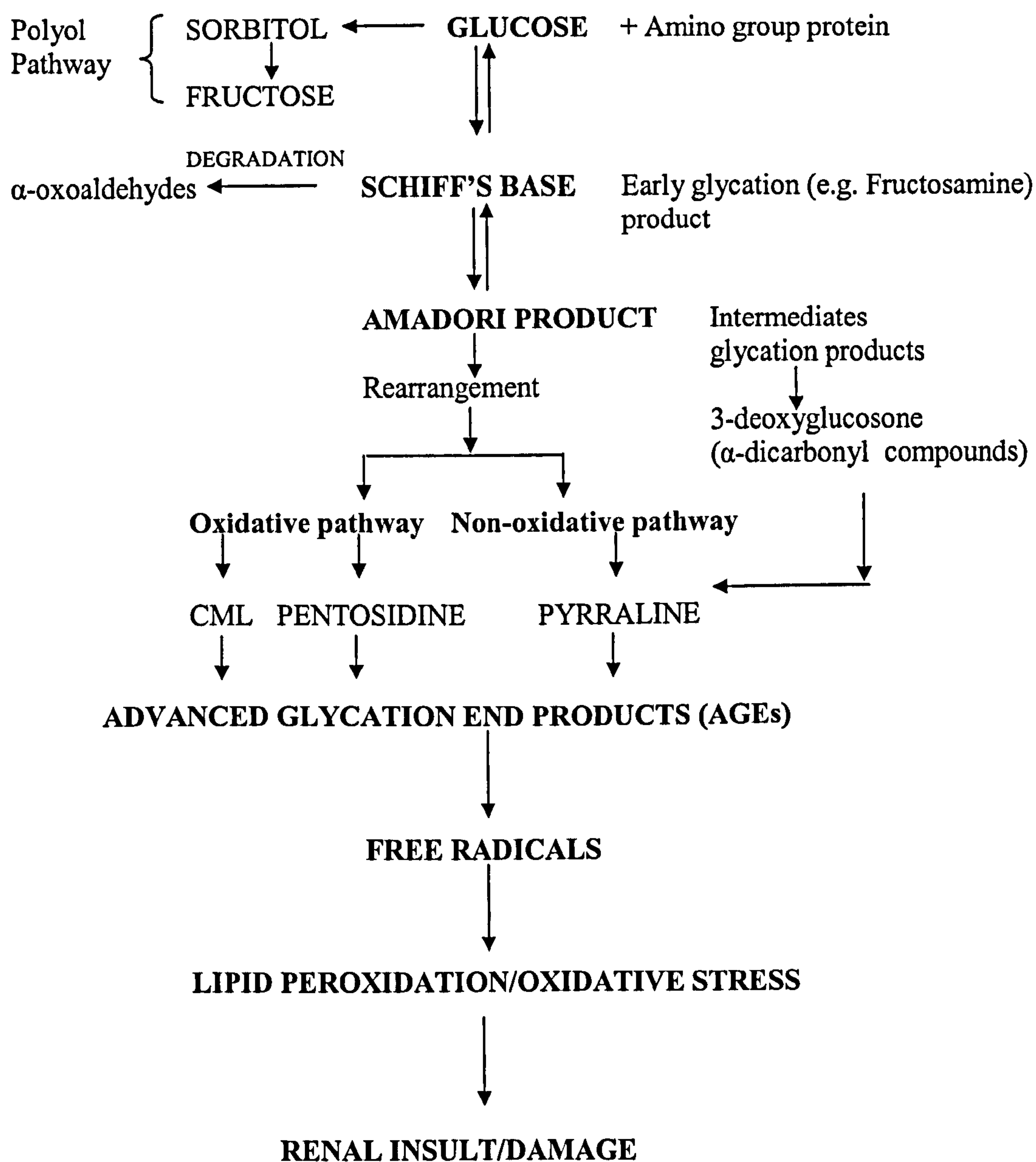
products to *N*<sup>ε</sup>-carboxymethyllysine, and the autoxidation of glucose, which produces superoxide radical anions, hydrogen peroxide and α-ketoaldehydes contribute to the generation of free radicals (Hunt *et al*, 1990). Several cell associated binding proteins for AGEs have been identified, including receptor for AGEs (RAGE) (Vlassara, 1996). The best characterised AGE receptor is RAGE, which is multiligand member of the immunoglobulin superfamily (Schmidt *et al*, 1996). The RAGE receptor probably acts as a scavenger and mediates intracellular signalling. Recently *in vitro* studies have demonstrated that AGE-RAGE binding on macrophages leads to free radical mediated oxidative stress and activation of the transcription factor NF-κB (Schmidt *et al*, 1994; Schmidt *et al*, 1996).



**Figure 1.3.7.1:** The glycation reaction of proteins in haemoglobin as an example

### 1.3.7.2 Polyol pathway

Hyperglycaemia associated with increased glucose metabolism can lead to the accumulation of sorbitol (Greene *et al*, 1988). Intracellular formation of sorbitol from glucose catalysed by aldose reductase (AR) is the first enzyme in the polyol pathway. In a hyperglycaemic situation, increased intracellular glucose results in its enzymatic conversion to the polyalcohol sorbitol, with concomitant decreases in NADPH. In the polyol pathway, sorbitol is then oxidized to fructose by the enzyme sorbitol dehydrogenase, with a stoichiometric reduction of  $\text{NAD}^+$  (Greene *et al*, 1988). Organ dysfunction in diabetes caused by the increased flux of glucose through the polyol pathway has been linked to the hyperglycaemia-induced increase in the  $\text{NADH}/\text{NAD}^+$  ratio, which is associated with the *de novo* synthesis of diacylglycerol (DAG) and the downstream stimulation of protein kinase C (PKC) activity. Disordered cellular metabolism or depletion of myo-inositol uptake, increased osmotic pressure and diminished  $\text{Na}^+/\text{K}^+$ -ATPase activity may also be contributory factors, for example the reduced glomerular hyperfiltration observed in an animal model of type 1 diabetes (Goldfarb *et al*, 1991). Furthermore, recently it has been suggested that oxidation of sorbitol by  $\text{NAD}^+$  increases the cytosolic  $\text{NADH}/\text{NAD}^+$  ratio, thereby inhibiting activity of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and subsequent increased concentrations of triose phosphates. Elevation of triose phosphate concentrations could increase formation of AGEs, and DAG thus activating PKC (Brownlee, 2001). The activation of PKC, through which hyperglycaemia stimulates extracellular matrix (ECM) production, is presumably due to increased *de novo* synthesis of DAG. The increase in the  $\text{NADH}/\text{NAD}^+$  ratio that results from the increased activity



**Figure 1.3.7.2** Glucose and AGEs formation pathways incorporating the polyol pathway.  
CML=*N*-ε-(carboxymethyl) lysine.



of the polyol pathway favouring this process. Increased *de novo* synthesis of DAG activates PKC both in cultured vascular, retinal and glomeruli cells in diabetic animals (Koya and King, 1998). However, increased *de novo* synthesis of DAG (Koya and King, 1998) and the effect of hyperglycaemia on PKC activation probably reflects increased dihydroxyacetone phosphate concentrations, resulting from inhibition of GADPH by free radicals. Hyperglycaemia may also activate PKC isoforms (PKC- $\alpha$  and PKC- $\beta$ ) indirectly through both ligation of AGE receptors and increased activity of the polyol pathway, presumably by increasing production of free radicals (Brownlee, 2001) thereby enhancing oxidative stress.

### **1.3.8 Biochemical markers of free radical mediated oxidative stress**

In the present study, appropriate biochemical markers were used in order to evaluate free radical by products and antioxidants in cohorts of both type 1 and type 2 diabetics as described below.

#### **Thiobarbituric acid reactive substances (TBARS)**

*In vivo* measurement of reactive free radicals is difficult because of their reactivity, short half-life, low concentration and complexity of products formed (Baynes and Thorpe, 1996). Consequently, measurements of thiobarbituric reactive substances are suggested to reflecting free radical generation and peroxidative damage. TBARS mainly consists of malondialdehyde (MDA), recognised end products of polyunsaturated fatty acids peroxidation. However, they are also formed during oxidative stress including DNA,

protein, or carbohydrates. In clinical and experimental studies, TBARS measurements have been used for evaluate lipid peroxide both in plasma (Kuyvenhoven and Meinders, 1999) and urine (Kosugi *et al*, 1993).

Increased TBARS concentrations in urine were observed in poorly control type 1 and type 2 diabetic subjects when compared to control subjects (Sato *et al*, 1979). Several studies have reported an increased susceptibility to lipid peroxidation in subjects with diabetes mellitus and diabetic nephropathy as measured by TBARS assay (Gallou *et al*, 1993; Rabini *et al*, 1994).

### Total Antioxidant Status

The antioxidant defense mechanisms provide protection from reactive free radical products, which cause peroxidation of lipids and destruction of biological molecules in the cell. Several methods (Whitehead *et al*, 1992; Miller *et al*, 1993) have been developed to determine the total antioxidant capacity of human serum or plasma because of the difficulty of measuring each individuals antioxidant components in the serum or plasma (Cao and Prior, 1998). Recently, the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) assay introduced by Miller *et al*, (1993) has been introduced. The assay based on the inhibition by antioxidants of the absorbance of the radical cation of 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) as described in Chapter 2.

Several studies have noted a decrease in total antioxidant status in the plasma of subjects with diabetes (Sundaram *et al*, 1996; Leonhardt *et al*, 1996; Srinivasan *et al*, 1997). Recently, Valublji *et al*, (2001) demonstrated reduced total antioxidant status in type 1 diabetes compared with non-diabetic control subjects. The reduction in total antioxidant status in diabetics has been associated with poor glycaemic control and duration of diabetes.

### Vitamin E ( $\alpha$ -tocopherol)

Vitamin E includes eight, naturally occurring compounds in two classes such as tocopherols and tocotrenols, and in each class there are  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ . The tocopherols are more active than tocotrenols, which have three double bonds in the side chain.  $\alpha$ -tocopherol is biologically the most active and widespread. One of the most characterised features of  $\alpha$ -tocopherol is its ability to prevent the initiation and propagation of lipid peroxidation by scavenging the free radicals contributing to the process by preventing oxidative damage to cell membrane thereby preserving structure and functional integrity of membranes and organelles. (Warren *et al*, 1992).

Several studies, particularly clinical prospective studies have demonstrated decreased plasma vitamin E concentrations in subjects with diabetes compared with value from non-diabetic control subjects (Salone *et al*, 1995). Furthermore, vitamin E concentrations were directly associated with LDL concentrations subjects with type 2 diabetes (Reaven *et al*, 1995).



## Glutathione peroxidase

In normal biochemical processes, superoxide is detoxified rapidly by the enzyme superoxide dismutase (SOD), leading to the formation of hydrogen peroxide, which is subsequently converted to water in the cytosolic compartment of the cells, through the action of the selenoenzyme glutathione peroxidase (GSH-Px) using glutathione as an essential cofactor. Glutathione peroxidases (EC 1.11.1.9) constitute a family of enzymes which are capable of reducing a variety of organic hydroperoxides and lipid hydroperoxides to the corresponding hydroxyl compounds, utilizing glutathione, and/or other reducing equivalents, thus protecting cells from free radical mediated oxidative damage. Furthermore, two types of glutathione peroxidases have been identified in the blood, namely classical cellular glutathione peroxidase in red blood cells and extracellular glutathione peroxidase in plasma (Brigelius-Flohe, 1999).

Several studies, including both human (Aaseth and Stoa-Birketvedt, 2000; Varvarovska *et al*, 2003) and experimental (Mukherjee *et al*, 1998) have reported significant depletion of glutathione peroxidase in diabetics associated with enhanced lipid peroxidation as a result of decreased scavenging of free radicals. In addition, a recent study reports a dramatic decrease in glutathione peroxidase activity, which occurred early in the course of chronic renal failure and associated with diabetic renal parenchymal destruction (Bonfont-Rousselt *et al*, 1997)

## Selenium

Selenium has been demonstrated to be essential for effective intermediate metabolism. Its biochemistry, toxicology and nutritional importance have been recently reviewed (Whanger, 1998). The antioxidant ability of selenium resides in the active side chain of the seleno-enzyme glutathione peroxidase. The importance of selenium as an essential trace element is probably due to its presence in this enzyme. Furthermore, selenium is generally present as either part of the metalloenzyme, glutathione peroxidase, or as selenoprotein P which function remain as yet unclear (Sheehan and Halls, 1999).

Several studies have reported a decreased selenium concentrations in subjects with type 1 and type 2 diabetes. In ketotic type 1 diabetic subjects, plasma selenium concentrations tended to be decreased in comparison with standard values and were not corrected by insulin treatment that normalised blood glucose concentrations (Faure *et al*, 1993). Furthermore, the effects of selenium on diabetic complications, an association between plasma selenium concentrations and high index of micro and macroangiopathy was observed in subjects with both type 1 and type 2 diabetes (Douillet *et al*, 1999).

### **1.3.9 Aims and objectives of the present study**

Evidence has accumulated indicating that hyperglycaemia associated with generation of free radicals and subsequent enhanced oxidative stress may play an important role in the pathogenesis of diabetic nephropathy. These processes are believed to reflect biochemical/pathophysiological changes in the diabetic kidney, resulting in both glomerular and tubular insult that may ultimately lead to the development of early diabetic nephropathy. However, the exact pathogenic processes, and the time needed to develop renal dysfunction involved in the development of diabetic nephropathy have not yet been clearly defined. Consequently, the overall aim of the present studies was to investigate the role of free radicals in the pathogenesis of diabetic nephropathy. Specific aims were as follows.

1. To investigate the role of hyperglycaemia and poor glycaemic control in promoting lipid peroxidation and subsequent oxidative stress in a cohort of diabetic subjects.
2. To establish any associations between estimates of lipid peroxidation, associated oxidative stress and early renal insult in the diabetic state.
3. To investigate any temporal relationship between disease duration, length of exposure to hyperglycaemia and altered carbohydrate and lipid metabolism in the diabetic state.
4. To investigate the role of antioxidant defence mechanisms with disease duration and any associations with renal insult and dysfunction in the diabetic state.
5. To investigate the use of sensitive renal biomarkers in the early recognition of diabetic renal insult and dysfunction, and any associations with lipid peroxidation, associated antioxidant defence mechanisms and glycaemic control.



## **CHAPTER TWO**

### **MATERIALS AND METHODS**

## **2. Materials and methods**

### **2.1 Clinical investigation protocol**

One of the main aims of the present studies was to investigate if the generation of free radicals and lipid peroxides, associated dyslipidaemia and renal insult was temporally related to the presence of hyperglycaemia. Consequently, length of disease duration rather than the type of diabetes present, was a major consideration in selecting study populations. However, this was recognised as being a significant limitation of the present study due to the heterogeneous nature of the type 2 diabetic state.

#### **2.1.1 Study design**

The subjects were patients undergoing routine clinical review in the out patients clinic at York Diabetes & Endocrine Centre, York District Hospital, York National Health Services Trust and diagnosed as type 1 or type 2 diabetics according to the WHO (1985) criteria. All patients volunteered and appropriate clinical information (age, gender, duration of disease, type of diabetes and current medication) was obtained from the diabetes assessment package. Both inclusion and exclusion criteria were adopted as follows.

1. All volunteers should be aged 18 and 68 years.
2. Diabetic subjects with evidence of renal disease characterised by frank proteinuria, hypertension with or without antihypertensive treatments, dyslipidaemia or cardiovascular disease, evidence of renal insufficiency, chronic urinary tract

infections, renal stones and renal tumors and any antioxidant supplementations were excluded from the study.

3. Informed consent would be obtained from all patients for the acquisition of human biological specimens, both blood and urine.
4. Age and gender-matched control subjects studied for comparative reasons were healthy individuals.

Sixty diabetic subjects were recruited and separated into two groups (both 20 subjects) according to duration of disease i.e. Duration of diabetes was defined as the time between diagnosis and initial visit for the present study. The two groups studied, in addition to a healthy non-diabetic control group were 1. Recently diagnosed diabetics (disease duration up to 2 years) and 2. Established diabetics (6-8 years disease duration). The study was reviewed and approved by both the De Montfort University Research Ethics Committee and York Heath Services Research Ethics Committee, and conducted under the guidance and supervision of Dr. Paul Jennings, Consultant Physician in diabetes and endocrinology, York Diabetes & Endocrine Center, York District Hospital, and Professor Paul Whiting, Professor of Biomedical Science at De Montfort University. The clinical characterization of these patients is given in Table 2.1.1



**Table: 2.1.1 Clinical characteristics of the study groups**

	Control Group/ Cont GP	Group 1/GP 1 (Recently diagnosed diabetics)	Group 2/GP 2 (Established diabetics)
n	20	20	20
Duration	--	≤ 2 years	6-8 years
Age (years; mean±SD)	41±17	40±18	40±18
Type 1/ Type 2	--	9/11	9/11
Male/Female	10/10	10/10	10/10
Insulin therapy	--	9	9
Metformin	--	5	5
Glibenclamide	--	2	2
Metformin and glibenclamide	--	4	4

### **2.1.2 Specimen collection, transportation and storage**

Appropriate sample acquisition, storage, and handling are essential, but frequently neglected, steps that precede biochemical investigations. However, it is crucial that all specimens to be used for experimental investigation have been collected from the appropriate patients using standardized protocols and placed in the appropriately labeled specimen collection tubes, and the stored in a manner that maintained specimen integrity.

In this study, fasting venous blood samples (10-12 hours) were obtained from selected diabetic subjects by a research nurse in the out patients clinic at York Diabetes & Endocrine Centre. Blood samples were collected in clean polypropylene tubes, labeled and immediately transferred to the Chemical Pathology/Clinical Chemistry laboratory for biochemical investigations. Although in most cases urine is an easier specimen to obtain than blood, collection requirements must also be strictly adhered. Furthermore, all urine specimens must be appropriately stored to minimise the risk of growth. In this study, morning mid stream urine specimens were collected in clean polypropylene tubes from previously selected diabetes subjects in the hospital. Urine specimens were labeled and immediately transferred to the Chemical Pathology/Clinical Chemistry laboratory for biochemical investigations. All specimens (blood and urine) were aliquoted into 2ml and storage at -20 °C unless otherwise stated. All frozen specimens were thawed completely and mixed well before the appropriate analysis was performed.

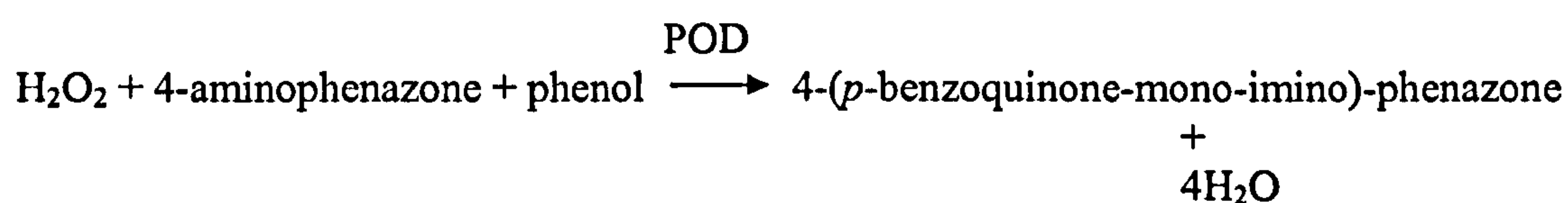
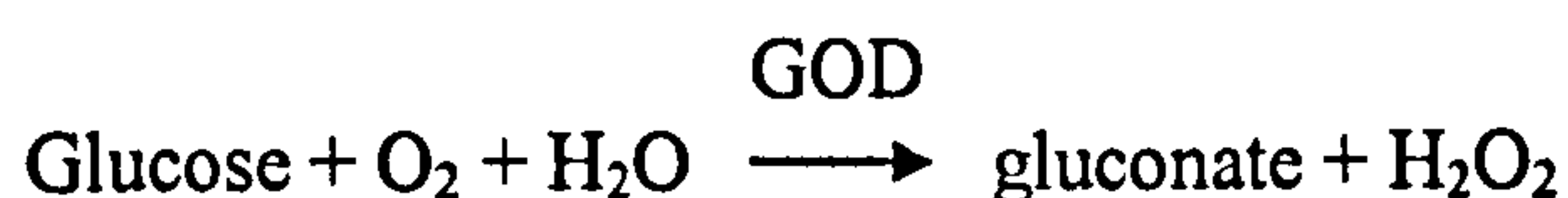
Various laws and regulations apply to the shipment and or transportation of biological specimens. It is very important factor that appropriate temperature must be maintained

during the transportation of the clinical specimens. Commercially available Styrofoam containers with dry ice were used for the transportation of all specimens (blood and urine) from York District Hospital to De Montfort University for biochemical analysis.

## 2.2 Metabolic biochemical markers investigation

### 2.2.1 Glucose analysis

Serum glucose concentrations were measured using the glucose oxidase method (enzymatic colorimetric) described by Tinder, (1969). Glucose is oxidized to gluconate by atmospheric oxygen in the presence of glucose oxidase (GOD). The indicator 4-aminophenazone and phenol are oxidized by the hydrogen peroxide forms in the presence of peroxidase (POD). The intensity of the colour produced is proportional to the glucose concentration in the sample. Finally, colour intensity was measured at 546 nm on a Hitachi 911 fully automated clinical chemistry analyser manufactured by Boehringer Mannheim /Roche Diagnostics Germany. The glucose reagent kit was supplied by the Boehringer Mannheim /Roche Diagnostics Germany. Results were expressed as mmol/l. The test principle of the glucose oxidase method is given below.





### **2.2.2 Fructosamine analysis**

Serum fasting fructosamine concentration was measured according to the colorimetric assay method described by Kruse-Jarres *et al*, (1989). Fructosamine (ketoamines) from the sample is present in an alkaline medium (reagent solution at pH 10.3) in the enol form, which reduces nitrotetrazolium-blue (NBT) to a formazan compound. The rate of formation of formazan is directly proportional to the concentration of fructosamine presence in the sample. The rate of reaction was measured photometrically at 546 nm on a Hitachi 911 fully automated clinical chemistry analyser (Boehringer Mannheim /Roche Diagnostics Germany). The fructosamine reagent kit was supplied by the Boehringer Mannheim /Roche Diagnostics Germany. Results were expressed as mmol/l.

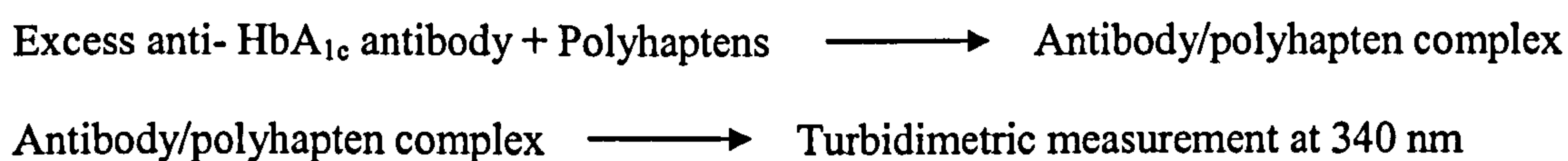
### **2.2.3 Glycosylated haemoglobin (HbA<sub>1c</sub>) analysis**

The HbA<sub>1c</sub> assay was based on the turbidimetric inhibition immunoassay (TINIA) as introduced by Karl, (1993). A commercially available reagent kit (Tina-quant HbA<sub>1c</sub>, Boehringer Mannheim /Roche Diagnostics Germany) was used to determine HbA<sub>1c</sub> % in the whole blood samples. The HbA<sub>1c</sub> kit was contained the following reagents.

Reagent 1 (R1): 2-morpholinoethane sulfonic acid (MES) buffer, tris (hydroxymethyl)-aminomethane [TRIS] buffer and HbA<sub>1c</sub> antibody (ovine serum).

Reagent 2 (R2): MES buffer, TRIS buffer and HbA<sub>1c</sub> polyhapten.

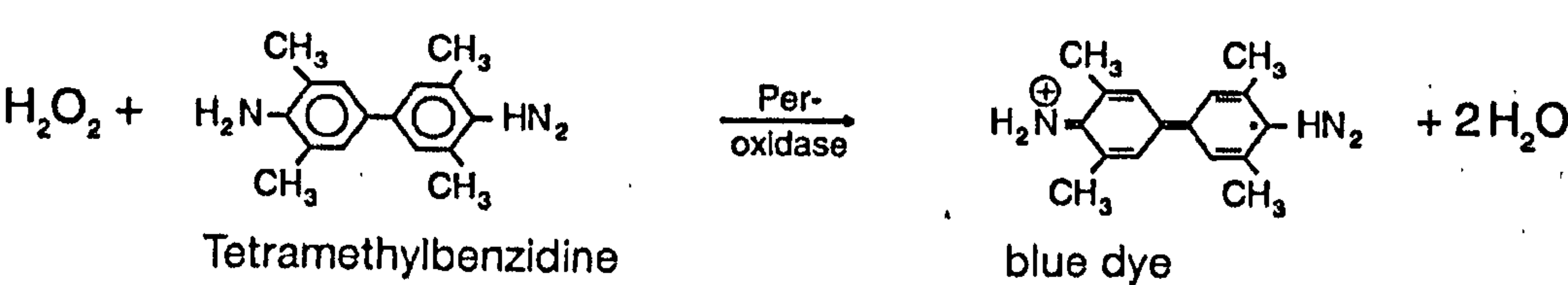
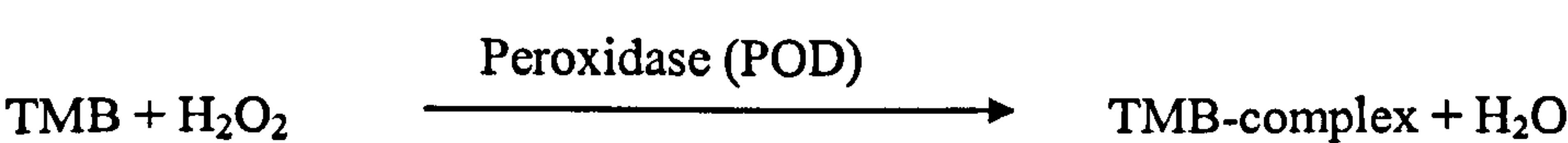
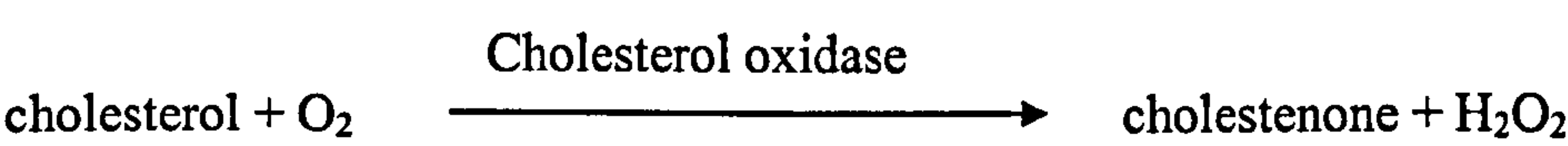
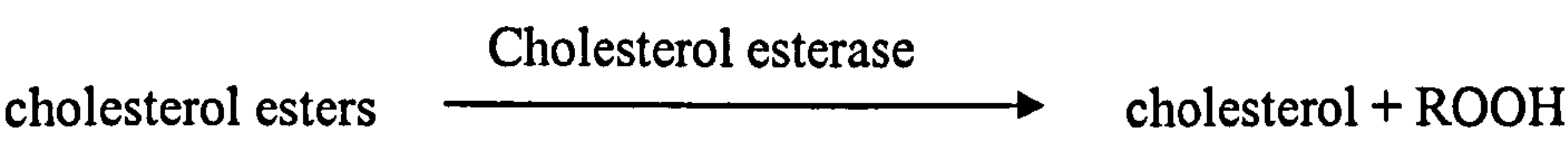
In brief, R1 was added to the blood samples to be measured. HbA<sub>1c</sub> in the sample reacts with the anti- HbA<sub>1c</sub> antibody to form soluble antigen-antibody complexes. Since the specific HbA<sub>1c</sub> antibody site was present only once on the HbA<sub>1c</sub> molecule, complex formation does not take place. After the addition of R2 which contained buffers and polyhapten the reaction was initiated. The polyhaptens react with excess anti- HbA<sub>1c</sub> antibodies to form an insoluble antibody-polyhapten complex which was measured turbidimetrically at 340 nm on a Hitachi 911 fully automated clinical chemistry analyzer (Boehringer Mannheim /Roche Diagnostics Germany). Results were expressed as percentage of HbA<sub>1c</sub>/total Hb. The HbA<sub>1c</sub> assay principle is given below



#### 2.2.4 Total cholesterol analysis

Serum cholesterol concentrations were measured by the method of Siedel *et al*, (1983). Cholesterol esters were first hydrolyzed into cholesterol and fatty acids in the presence of cholesterol esterase. Free cholesterol was then oxidized into cholestenone by atmospheric oxygen in the presence of cholesterol oxidase, the reaction also producing hydrogen peroxide. The latter compound then oxidised an incorporated indicator namely, 3,3',5,5'-tetramethylbenzidine (TMB), in the presence of peroxidase to produce a blue coloured complex. The concentration of complex was finally determined by a microprocessor-controlled reflectance photometer at 642 nm. The microprocessor-controlled reflectance

spectrophotometer (“REFLOTRON” semi automated dry chemistry analyzer, manufactured by Boehringer Mannheim /Roche Diagnostics Germany) and dry chemistry reagent carrier test strips (REFLOTRON test strips produced by Boehringer Mannheim /Roche Diagnostics Germany) were used for the determination of all lipid parameters such as total cholesterol, triglycerides and HDL in this study. Results were expressed as mmol/l. The reaction principle is given below.

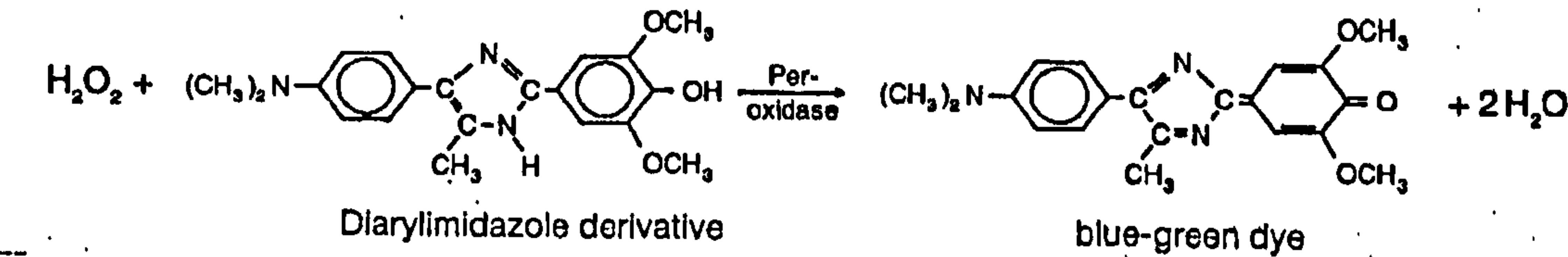
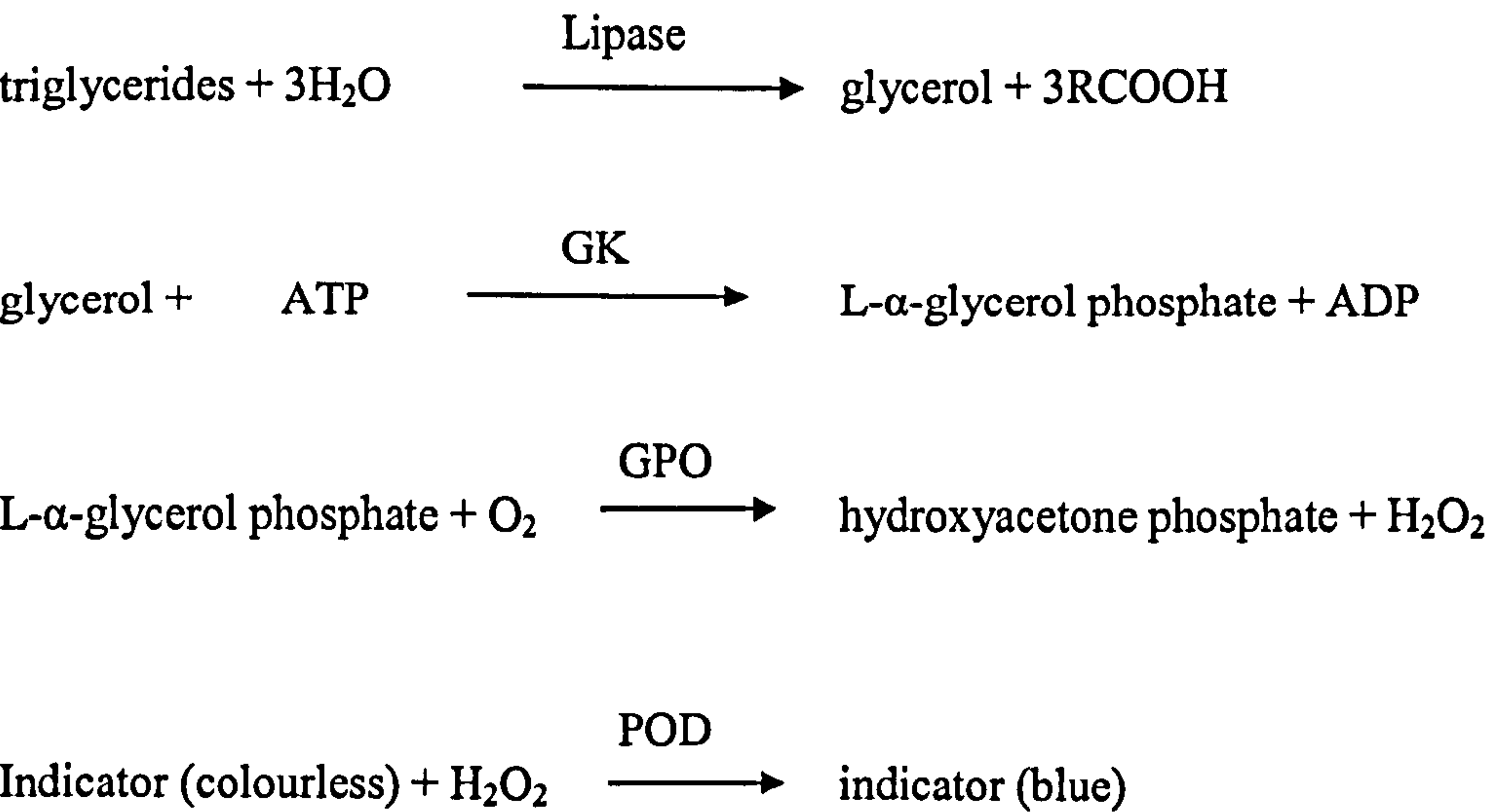


(The indicator reaction)



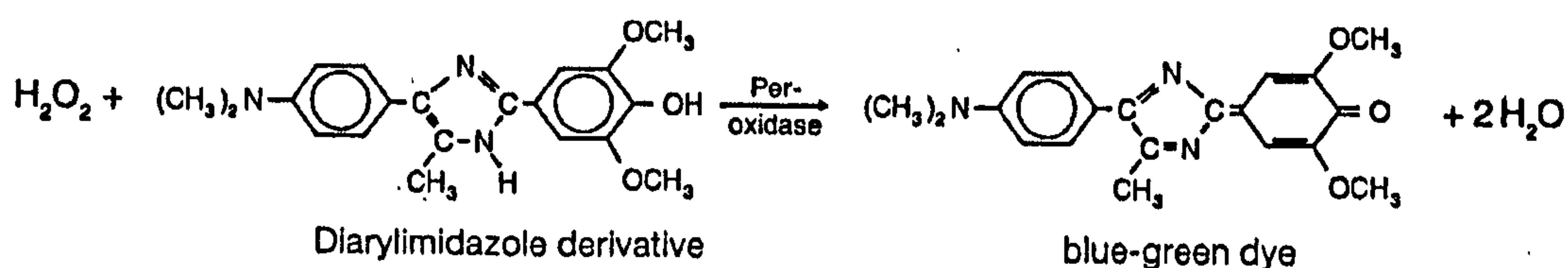
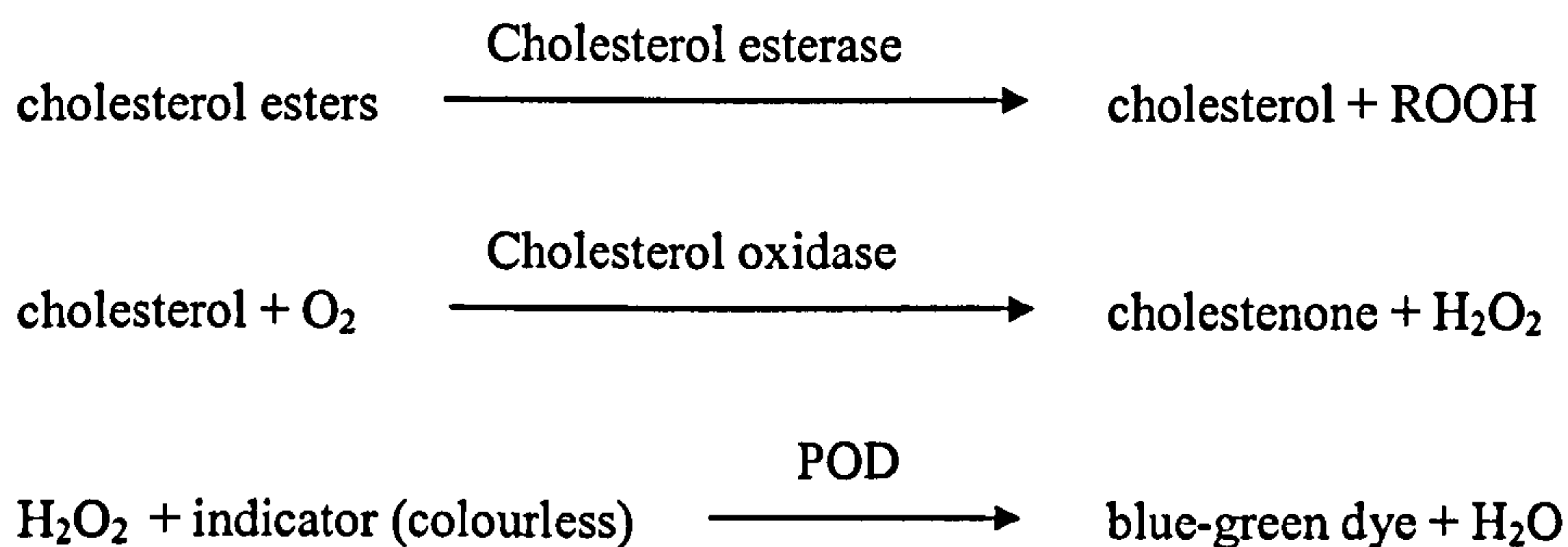
2.2.5 Triglycerides analysis

Serum triglycerides were measured according to the method described by Nagele *et al*, (1984). The triglycerides were first hydrolysed into glycerol and free fatty acids by lipase. The glycerol was then phosphorylated into L-α-glycerol phosphate by glycerokinase (GK) and adenosine triphosphate (ATP) in presence of Mg<sup>2+</sup>ions. The glycerol phosphate was then oxidised by atmospheric oxygen into hydroxyacetone phosphate and hydrogen peroxide in the presence of L-α-glycerol phosphate oxidase (GPO). Finally, the blue colour of the oxidised indicator, which becomes deeper coloured with time, is determined using a “REFLOTRON” dry chemistry analyzer at 642 nm. Results were expressed as mmol/l. The reaction principle is given below.



### 2.2.6 High density lipoprotein (HDL) cholesterol analysis

Serum HDL concentrations were measured by the method of Siedel *et al*, (1983). The enzyme cholesterol esterase catalyses the cleavage of cholesterol esters into cholesterol and the corresponding fatty acid. In the presence of oxygen, free cholesterol is oxidized by means of cholesterol oxidase to produce chlestenone and hydrogen peroxide. The hydrogen peroxide forms converts the reduced form of the indicator 4-(4-dimethylaminophenyl)-5-methyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-imidazole dihydrochloride into blue (oxidized) form, provided peroxidase (POD) is present. The concentration of blue dye is proportional to the concentration of HDL in the sample and was determined by "REFLOTRON" dry chemistry analyzer at 642 nm. Results were expressed as mmol/l. The reaction principle is given below.



**(The indicator reaction)**

### 2.2.7 Low density lipoprotein (LDL) cholesterol estimation

#### The Friedewald Equation

In the most widely used indirect method, total cholesterol, triglycerides and HDL cholesterol are measured and LDL cholesterol is calculated from the primary measurements using the empirical equation of Friedewald *et al*, (1972). In this study LDL concentration of the samples were estimated by the Friedewald equation as given below. Results were expressed as mmol/l.

$$[\text{LDL cholesterol}] = [\text{Total cholesterol}] - [\text{HDL cholesterol}] - \frac{[\text{Triglycerides}]}{2.2} \quad (\text{mmol/l})$$



## **2.3. Lipid peroxidation and plasma antioxidant investigations**

### **2.3.1 Urinary Thiobarbituric Acid Reactive Substances (TBARS) analysis**

The most widely used method for the detection of lipid peroxidation is the TBA reaction with malondialdehyde (MDA) resulting in the formation of a MDA-TBA complex. This complex could be separated from other TBA-reactive compounds by both chromatography and spectrofluorimetry. Urine TBARS were analysed using a fluorimetric technique introduced by Yagi, (1976) and subsequently modified by Wasowicz *et al*, (1993).

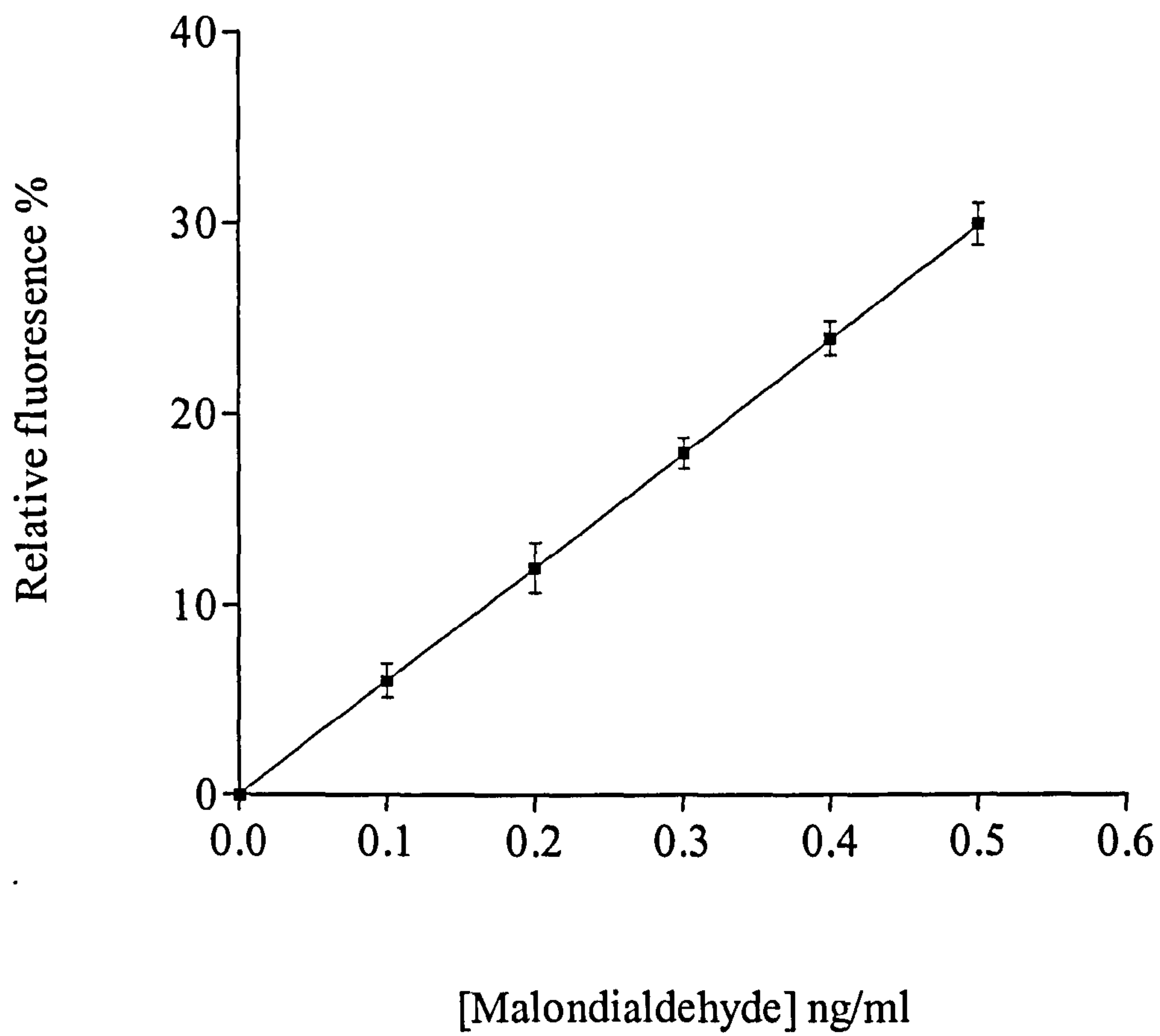
.Reagents were prepared using double distilled deionised water in order to prevent iron contamination, which may cause an arteactual increase in the final fluorescent product. The TBA reagent was prepared by mixing thiobarbituric acid 0.67% (w/v) with an equal volume of glacial acetic acid. A stock solution of MDA (0.5 nmol/ml) was prepared by hydrolysing tetramethoxypropane (103 µl; MW 164.2, 0.997 g/ml) with water to a final volume of 250 ml and then dissolving 50 µl of this solution in another 250 ml of water. Standards (0 to 0.5 nmol/ml) were prepared by placing the appropriate quantity of stock solution in a glass tube and adding water to a final volume of 1 ml. Previously aliquoted and stored (at -70°C) urine specimens were brought up to room temperature, reconstituted and 100 µl pipetted into a separate glass tubes and water (0.9 ml) added. TBA reagent (1ml) was then added to both standards and specimens and they were placed in a water bath and heated for 1 hour at 95–100 °C After the samples cooled, 5 ml of butanol was added to each tube and after mixing, the tubes centrifuged at 1500 r.p.m. for

10 minutes. Finally, the butanol layer was read using a fluorimeter (Perkin-Elmer LS-5, UK) with excitation at 515 nm and emission at 546 nm and a standard curve was constructed as shown in Figure 2.3.1. The concentration of the unknown was then calculated. All standards and specimens were analysed in triplicate and all chemicals for this assay were obtained from Sigma-Aldrich Ltd, UK.

### **2.3.2 $\alpha$ -tocopherol (vitamin E) analysis**

$\alpha$ -Tocopherol was measured by the method of Macaulay, (1999) using a reverse phase high performance liquid chromatography (HPLC) system (Shimadzu Class-VP, Shimadzu Corporation, Japan).

An internal standard was added to the plasma samples, which were then extracted and loaded onto the column. Both  $\alpha$ -tocopherol and the internal standard,  $\alpha$ -tocopherol acetate, have polar attraction and specificity for the column and are therefore retained on the column before being eluted and detected. The retention times for  $\alpha$ -tocopherol and  $\alpha$ -tocopherol acetate were determined by observing the times for peaks eluting after loading pure standards. A standard  $\alpha$ -tocopherol solution was prepared by dissolving 20mg of  $\alpha$ -tocopherol (Sigma-Aldrich, UK) in 100ml of HPLC grade ethanol (Fisher Chemical, UK) (Standard Solution 1). Solution 1 was then further diluted 1 in 20 with ethanol to form solution 2. Maximum absorption of standard solution 2 was observed and confirmed at 292 nm by using a scanning spectrophotometer (UNICAM UV1) without any detectable contamination.



**Figure 2.3.1** Typical standard curve for TBARS assay. Results are expressed as mean  $\pm$  SEM for triplicate determinations.



The concentration of standard solution 1 (3.44mmol/l) was determined using the Beer-Lambert law. 146µl of standard solution 1 was made up to 10ml with pooled serum. Both spiked and non-spiked serum samples were aliquoted and frozen at -20 °C. For each run, a sample of spiked and non-spiked pooled serum was measured. The internal standard was prepared by dissolving approximately 14g of α-tocopherol acetate (Sigma-Aldrich, UK) in 50ml of ethanol. This solution was scanned and a single peak obtained indicating again no significant contamination.

Plasma samples (100µl) (spiked pool/non-spiked pool) were pipetted into a 1.5ml polypropylene tube and 100µl of α-tocopherol acetate internal standard was added. After vortex mixing for 20 seconds, 400µl of hexane (HPLC grade, Fisher Chemicals, UK) was added and, after further vortexing for 60 seconds, the tubes were centrifuged and the hexane layer separated. The sample were then dried under a stream of nitrogen, reconstituted with 50µl methanol (HPLC grade, Fisher Chemicals) and analysed.

A stainless steel column (C18: 5µl, 150 mm x 4,6 mm/Phenomenex UK) was used with the HPLC system and HPLC grade methanol was used as a mobile phase. The mobile phase was pumped through the column at 1.5ml per minute and the injection volume was 20µl. The retention time for α-tocopherol was 3.5 minutes and that for α-tocopherol acetate 4.4 minutes, determined from the analysis of standard solutions. Standards and samples were analysed and the areas under the respective peaks obtained. All standards and specimens were analysed in triplicate. A typical standard curve is given in Figure: 2.3.2 and the concentration of α-tocopherol in samples was calculated as detailed given below.

$$\text{Concentration} = \frac{\text{Absorbance}}{\text{Extinction coefficient} \times \text{Path length}} \quad (\text{from the Beer-Lambert Law})$$

$$\begin{aligned} \text{Extinction coefficient for } \alpha\text{-tocopherol} &= 75.8 \text{ for a 1\% solution} \\ &\text{(i.e. 1g in 100ml in a 1cm lightpath)} \\ \text{Pathlength} &= 1\text{cm} \end{aligned}$$

$$\text{Mean absorbance of standard solution} = 0.561$$

$$\begin{aligned} \alpha\text{-tocopherol in standard solution 1} &= \frac{20 \times 0.561}{75.8 \times 1} = \frac{0.148\text{g}}{100\text{ml}} = 3.44\text{mmol/l} \\ \text{(MW 430.7)} & \end{aligned}$$

$$146\mu\text{l made up to 10ml with pooled serum} = 50 \mu\text{mol/l}$$

$$\frac{\text{Area } \alpha\text{-tocopherol in spiked pool}}{\text{Area internal standard in spiked pool}} = R_s$$

$$\frac{\text{Area } \alpha\text{-tocopherol in patient sample}}{\text{Area internal standard in patient sample}} = R_p$$

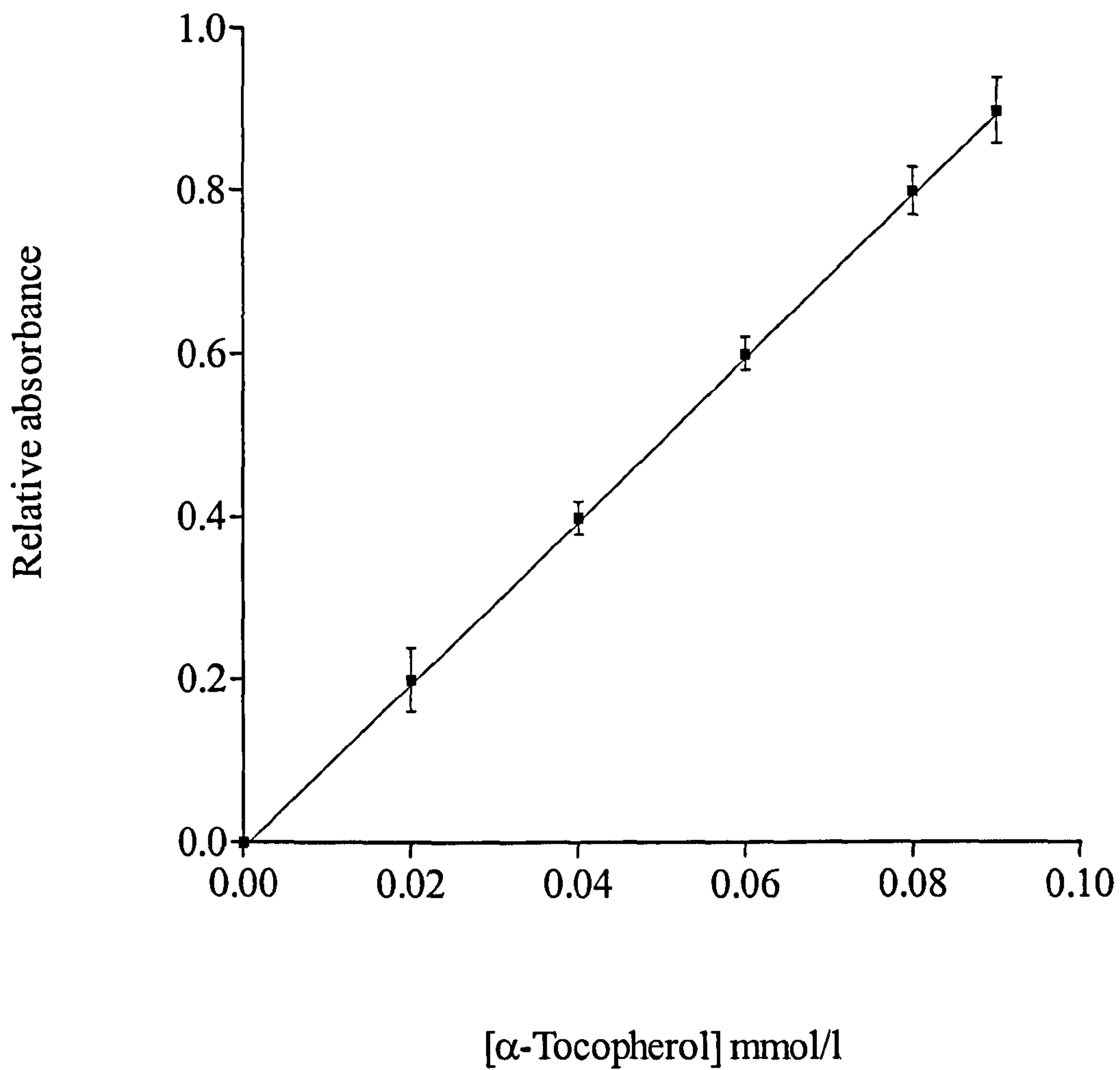
$$\frac{\text{Area } \alpha\text{-tocopherol in unspiked pool}}{\text{Area internal standard in unspiked pool}} = R_u$$

The concentration of the internal standard is the same in all three samples

The difference between the spiked and unspiked pool  $(R_s - R_u) = 50 \mu\text{mol/l}$

Therefore  $1\mu\text{mol/l} = R_s - R_u/50$  and the patients sample concentration can be calculated.

$$\text{Concentration of } \alpha\text{-tocopherol in the patient sample} = R_p \times (R_s - R_u)/50$$

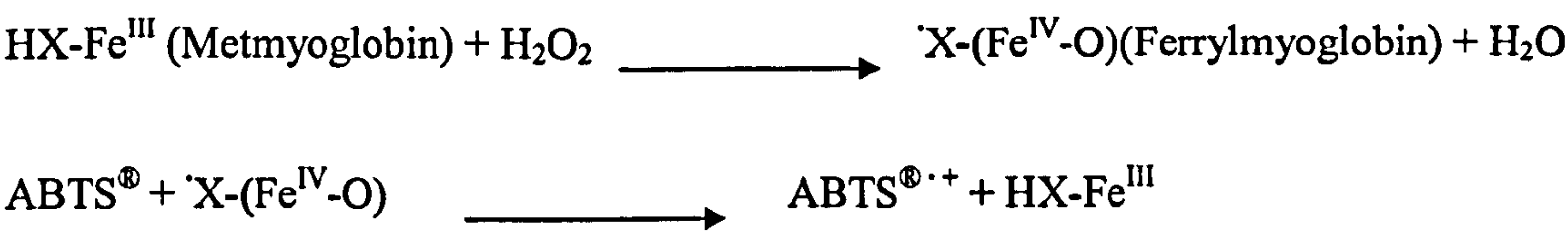


**Figure 2.3.2** Typical standard curve for the α-tocopherol (vitamin E) assay. Results are expressed as mean ± SEM for triplicate determinations.



**2.3.3. Total antioxidant status analysis**

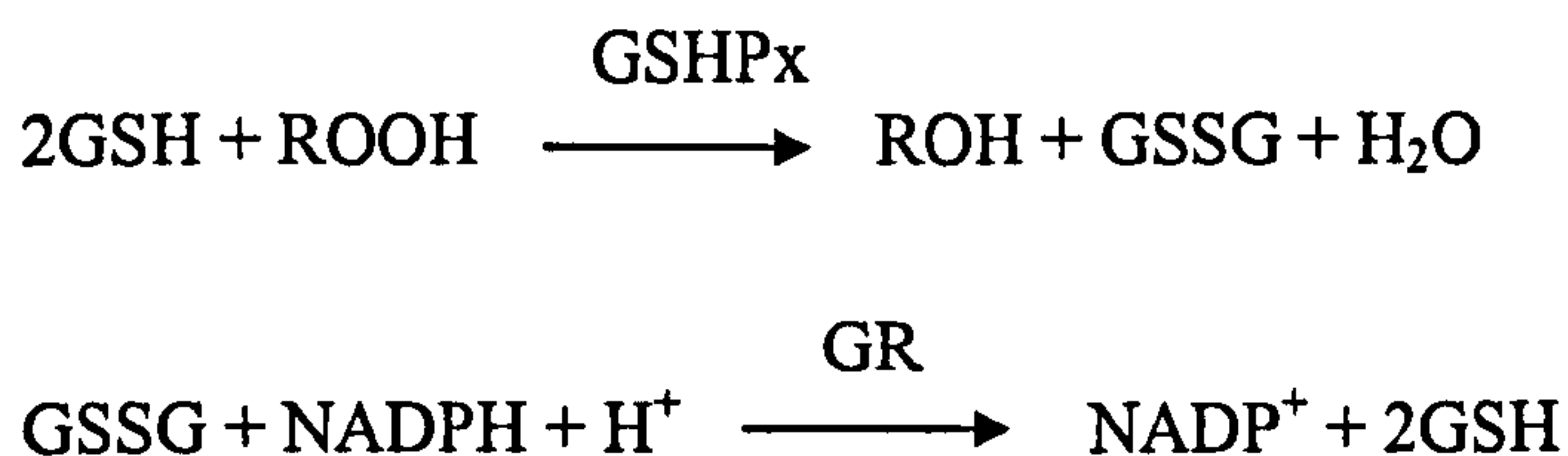
The total antioxidant status assay method described by Miller *et al*, (1993) was used in the present studies. Plasma total antioxidant status was measured using a commercial reagent kit (Randox Laboratory, UK). The assay principle is that metmyoglobin reacts with hydrogen peroxide to form the radical species, ferrylmyoglobin. A chromogen solution such as 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS<sup>®</sup>) is incubated with the ferrylmyoglobin to produce the radical cation species ABTS<sup>+</sup>. This has a relatively stable blue green colour, which was measured at 600 nm using a Hitachi 911 fully automated clinical chemistry analyzer. Antioxidants in the sample cause suppression of this colour production to a degree that was proportional to their concentration in the sample. Results were expressed as mmol/l. The reaction principle is given below.



**2.3.4. Glutathione peroxidase (GSHPx) (EC.1.11.19) analysis**

Glutathione peroxidase was measured by the method of Paglia and Valentine, (1967). In brief, glutathione peroxidase catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant

oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance at 340 nm was measured using a Hitachi 911 fully automated clinical chemistry analyzer. A commercial reagent kit (Randox Laboratory, UK) was used for the determination of glutathione peroxidase in this study. The reaction principle is given below.



### 2.3.5 Selenium analysis

Selenium concentrations in plasma were determined by a Varian model SpectrAA-880 Zeeman graphite furnace atomic absorption spectrometer (GFAAS, Varian, UK) with software SpectrAA-880 following the method of Knowles and Brodie, (1988). In brief, plasma samples were diluted 1/10 by using a sample diluent. The sample diluent was prepared by dissolving 0.56g of ascorbic acid (BDH,UK) 0.88 ml of TritonX-100 (BDH, UK), 1 ml of Antifoam B (Sigma-Aldrich, UK) and 0.1 ml of hydrochloric acid (Sigma-Aldrich, UK), made up to 1.0 L with deionized water. Then 20 µl of plasma sample was injected into the graphite furnace with a modifier solution (500 mg palladium, 0.05 % Triton X-100 and 0.1% Antifoam B). By using the auto sampler facility of the GFAAS the calibration curve was prepared from the stock solution of selenium (1 mg/L) by mixing the appropriate volume. The hollow cathode lamp from selenium was operate at 20 mA and absorbance was measured at 196 nm. The analysis was performed in triplicate and results are expressed as µmol/l.

## **2.4. Renal biochemical markers investigation**

### **2.4.1 Serum and urine creatinine analysis**

Serum and urine creatinine concentrations were measured by the method of Heinegard and Tiderstrom, (1973) a reaction first described by Jaffe in 1886. Creatinine in an alkaline solution reacts with picric acid to form a coloured (orange-red) complex. The amount of the complex formed is directly proportional to the creatinine concentration in the sample. A commercial reagent kit obtained from Boehringer Mannheim/Roche Diagnostics, Germany and measured at 546 nm using a Hitachi 911 fully automated clinical chemistry analyzer (Boehringer Mannheim/Roche Diagnostics, Germany). Results were express as  $\mu\text{mol/l}$  for both serum and urine.

### **2.4.2 Microalbuminuria analysis**

As previously described, the occurrence of microalbuminuria is an early feature of diabetic nephropathy and can be regarded as the first marker of impaired glomerular function in subjects with either type 1 and type 2 diabetes mellitus. Albuminuria analysis can be performed according to two methods including semi quantitative and quantitative. However, in the present study quantitative assay was performed as detailed below.



## Quantitative analysis

Quantitative albumin determination was based on an immunoturbidimetric assay (Multicentre study 1991) as initially introduced by Hofmann and Guder, (1989). A ready-made reagent kit (Tina-quant<sup>®</sup> Albumin) obtained from Boehringer Mannheim/Roche Diagnostics, Germany was used in the present study. It contained two reagents (reagent 1 and reagent 2) as described below.

Reagent 1/R 1(buffer): TRIS buffer, 50mmol/l. pH 8.0, EDTA 3.7 g/l

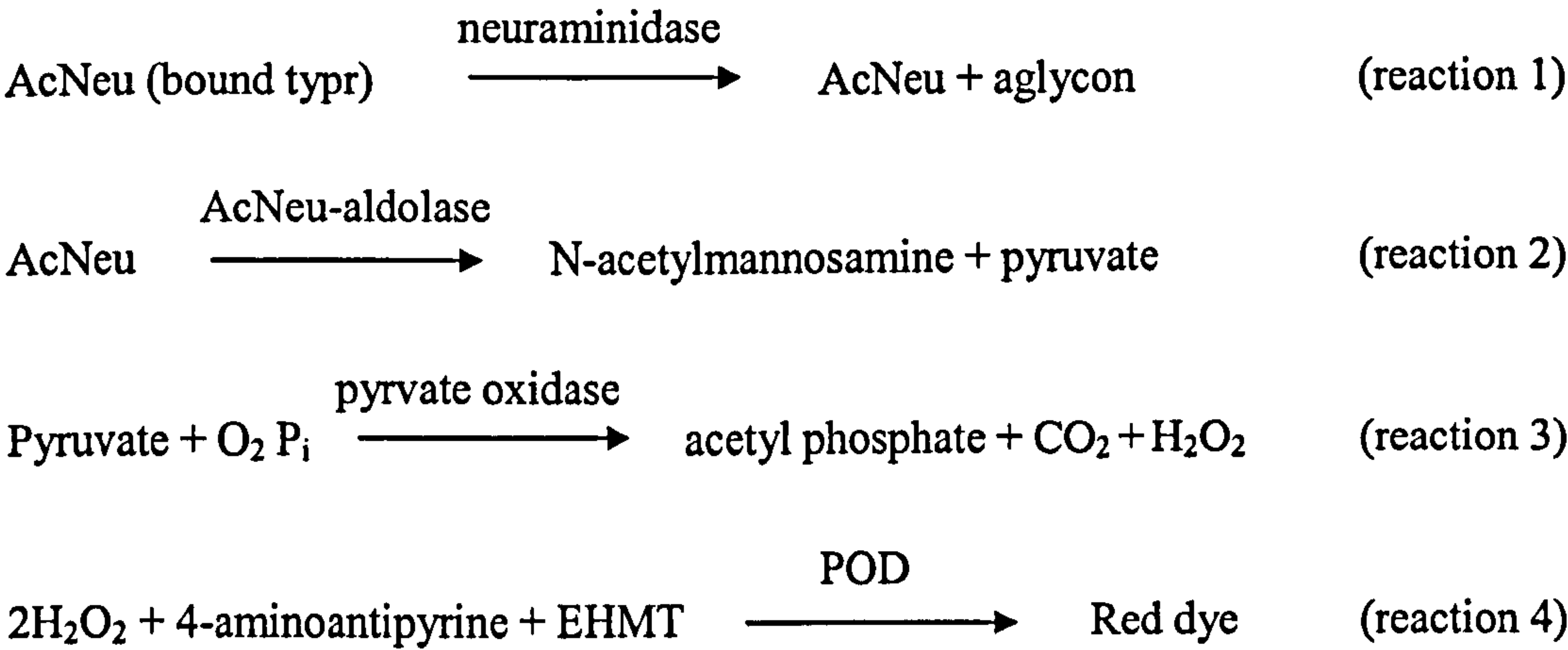
Reagent 2/R 2: Polyclonal anti-human albumin antibodies (sheep); TRIS buffer  
100mmol/l, pH 7.2

Initially, reagent R 1 (100 µl) was mixed with the urine specimen (20 µl) and then subsequently. The addition of reagent R 2 (150 µl) (anti-albumin antibodies) initiated the reaction. Anti-albumin antibodies react with the antigen in the urine specimen to form antigen-antibody complexes which, following agglutination, were measured turbidimetrically at 340 nm using a Hitachi 911 fully automated clinical chemistry analyzer. Results were expressed as mg/l.

### **2.4.3 Serum sialic acid analysis**

Serum sialic acid concentrations were measured by the method of Zoppi, (1985). The glycosidically bound sialic acid is hydrolysed by neuraminidase to release free N-acetylneuraminic acid (AcNeu) (reaction 1). In the presence of AcNeu-aldolase, AcNeu

is cleaved into N-acetylmannosamine and pyruvate (reaction 2). The pyruvate formed is oxidized in the presence of  $\text{MgCl}_2$ , flavine adenine dinucleotide (FAD) and thiamin pyrophosphate (TPP) by pyruvate oxidase to form acetyl phosphate,  $\text{CO}_2$  and  $\text{H}_2\text{O}_2$  (reaction 3). The amount of  $\text{H}_2\text{O}_2$  formed, which is equivalent to the free N-acetylneuraminic acid present, is converted by peroxidase (POD) in the presence of 4-aminoantipyrine (4-AAP) and N-ethyl-N-2-hydroxyethyl-3-toluidine (EHMT) to form a red coloured product (reaction 4), which absorbance was measured at 550 nm using a Hitachi 911 fully automated clinical chemistry analyzer. Results were expressed as mmol/l.



#### 2.4.4 Serum Cystatin C analysis

The cystatin C assay was based on the method of the turbidimetric immunoassay introduced by Kyhse-Andersen *et al* (1994). A commercial reagent kit (DAKO Cystatin C PET) was obtained from DakoCytomation Ltd, UK. It contained polystyrene particles of uniform size, chemically coupled with a rabbit antibody against human

Cystatin C. A reaction between these immunoparticules and Cystatin C in a patient specimen results in the formation of agglutinates and a concomitant changes in the absorbance measured at 340 nm. Again, a Hitachi 911 fully automated clinical chemistry analyzer was used for the analysis. Results were expressed as mg/L.

#### **2.4.5. Urine N-acetyl- $\beta$ -D-glucosaminidase assay/ $\beta$ -NAG (EC. 3. 2. 1. 30)**

$\beta$ -NAG activity in urine was measured by the method of Whiting, (1979) which was adapted from the method of Dance *et al*, (1969). The assay was based on the cleavage by  $\beta$ -NAG of the novel substrate 4-methylumbelliferyl-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside, a non-fluorescent compound to release 4-methylumbelliferone (4-MU). 4-MU fluoresces at a pH greater than 10, and consequently the reaction was terminated by the addition of an alkaline glycine buffer, pH 10.4. The final absorbency of 4-MU is obtained spectrophotometrically (UNICAM 8625 UV/VIS, UK) at a wavelength of 360 nm. The following buffer solutions were used in the assay as detailed below.

- i. Trisodium citrate (TSC) buffer (assay buffer): trisodium citrate, 20 mM, pH 4.3 (with concentrated HCl).
- ii. Glycine buffer (stop solution): glycine, 0.5M adjusted pH 10.4 (with 5M NaOH).
- iii. Standard 4MU solution: 3mg of 4-methylumbelliferone (4-MU) in 1ml ethanol made up to 100ml with TSC buffer.
- iv. Substrate: 40mg of 4-methylumbelliferyl-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside was dissolved in 50ml of TSC buffer.

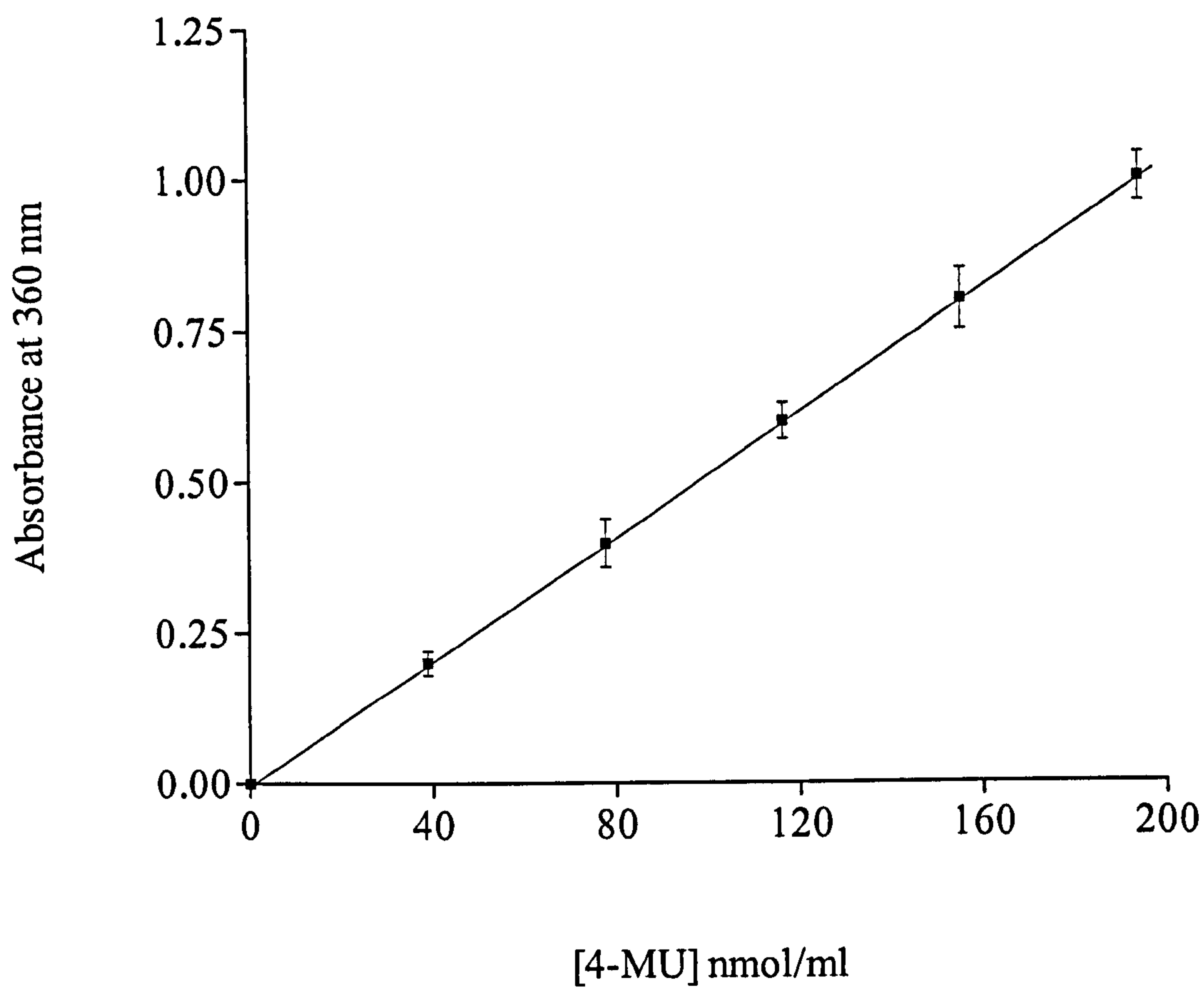


The following procedure was adopted in order to determined NAG activity in the diabetic and control urine specimens. A standard curve was constructed (Figure 2.4.5), specimens were analysed in triplicate and results were expressed as units/mmol urinary creatinine. All chemicals were used in this analysis were obtained from Sigma-Aldrich, UK.

Sample (Test)	Blank
0.1ml urine	0.1ml urine
0.65ml TSC buffer	0.65ml TSC buffer
_____	2ml glycine buffer
Incubate for 10 minutes at 37°C	
0.25ml substrate	0.25ml substrate
Incubate for 30 minutes at 37°C	
2ml glycine buffer	_____
Read optical density at 360 nm	

Calculation:  $E_{Corrected} = E_{Test} - E_{Blank}$

The amount of 4-MU released was determined from the standers curve. The activity was expressed as nmol/ml/hr = μmol/l/hr = unit of enzyme activity



**Figure 2.4.5** Typical standard curve for 4-methyl umbelliferone. Results are expressed as mean  $\pm$  SEM for triplicate determinations.

#### 2.4.6 Urine alanine amino peptidase (AAP) analysis ( $\alpha$ -aminoacyl-peptide hydrolase, EC 3.4.11.2)

Urine alanine amino peptidase activity was measured by the method of Jung and Scholz, (1980). The enzyme activity was determined in the presence of the model substrate L-alanine-4-nitroanilide hydrochloride which is hydrolysed by AAP to release the coloured compound 4-nitro aniline which absorbs maximally at 405 nm as measured by a UNICAM UV1 (UK) double-beam spectrophotometer. The reaction principle as given below.



Urine samples were prepared by using PD-10 Sephadex G-25M columns. Previously aliquoted and centrifuged urine 2ml was added to the column and the eluent drained and discarded. Collection of the filtered urine was obtained following the addition of 4ml of NaCl to the column. Then two solutions (solution 1 and solution 2) were prepared in order to determine AAP activity in urine. Solution 1 contained 59 mmol of tris adjusted to pH 7.8 with HCl and solution 2 contained 26 mmol of alanine-4-nitroanilide hydrochloride dissolved in solution 1 (250 ml). Then 1 ml of solution 1 was mixed with 0.2 ml of urine, and allowed to equilibrate at 37°C for 15 minutes.

The assay was initiated by the addition of 0.1 ml of solution 2 and the enzyme activity was determined kinetically by measuring the increase in optical density over a five minute period at 405 nm.



Calculation:

$$K = (TV/SV) \times 10^6 \times (1/E)$$

K = calibration factor

TV = final reagent volume with include sample volume

SV = sample volume

E = molar extinction coefficient factor of 4-nitroaniline = 9620

$$K = (1.3/0.2) \times 10^6 \times (1/9620) = 675.7$$

Optical density (OD) x K = IU

AAP activity was expressed as IU/mmol urinary creatinine

#### **2.4.7. Urine $\alpha$ 1-microglobulin analysis**

Urine  $\alpha$ 1-microglobulin concentration was measured using the method described by Kurrle-Weittenhiller and Engel, (1992), and based on an immunoturbidmetric assay. A commercially available ready-made reagent kit (Tina-quant<sup>®</sup>  $\alpha$ 1-microglobulin, Boehringer Mannheim/Roche Diagnostics, Germany) was used. The kit contained two reagents (R 1 and R 2). R1 mainly consisted of 35 mmol of acetate buffer at pH 5.3, while R2 contained a polyclonal anti-human  $\alpha$ 1-microglobulin antibody (sheep) and 50 mmol of acetate buffer at pH 5.3. Initially, reagent R1 (100  $\mu$ l) added to the urine specimen (20  $\mu$ l) mixed and then reagent R2 (150  $\mu$ l) was added start the reaction.  $\alpha$ 1-Microglobulin antibodies react with antigen in the urine specimen to form an antigen/antibody complex which, after agglutination, was detected tuibidimetrically using a Hitachi 911 fully automated clinical chemistry analyzer.

#### 2.4.8. Urine $\beta$ 2-microglobulin analysis

$\beta$ 2-microglobulin in urine was measured by the method of Junge *et al*, (1996). The method was based on a immunoturbidmetric assay. A commercial reagent kit was obtained from Roche Diagnostics Germany (Tina-quant<sup>®</sup>  $\beta$ 2-microglobulin, Boehringer Mannheim/Roche Diagnostics, Germany) and it contained the following reagents.

Reagent 1 (R1): TRIS/HCl buffer 23 g/l, pH 8.7; NaCl 19 g/l, EDTA 2 g/l

Reagent 2 (R2): latex particles coated with polyclonal anti-human  $\beta$ 2-microglobulin antibody (rabbit) 0.5 g/l

In brief, reagent R1 (100  $\mu$ l) was mixed with the urine specimen (20  $\mu$ l) and then addition of reagent R2 (150  $\mu$ l) initiated the reaction.  $\beta$ 2-microglobulin antibodies react with antigen in the urine specimen to form an antigen/antibody complex which, after agglutination, was detected turbidimetrically using a Hitachi 911 fully automated clinical chemistry analyzer.

#### **2.4.9 Urine alpha glutathione-S-transferase ( $\alpha$ GST) and pi glutathione-S-transferase ( $\pi$ GST) analysis**

The determination of both  $\alpha$ GST and  $\pi$ GST was based on a quantitative enzyme immunoassay. The analytical procedure was similar for both parameters and based on the sequential addition of urine sample, enzyme-conjugate and substrate to Microassay (96-well) plates pre-coated with anti-  $\alpha$ GST-IgG/  $\pi$ GST-IgG antibodies. The resultant colour intensity was proportional to the activity of GST present in the urine sample.

The  $\alpha$ GST and  $\pi$ GST activity in urine was measured using the method described by Hassett and Doyle, (1995) and Maning, (1995) respectively. Commercial reagent kits (Biotrin International Ltd, Dublin, Ireland) were used and the following components were included in the reagent kit.

- i. Antibody coated Microassay plate: 96-well plates (12x8 strips) pre-coated with IgG directed against  $\alpha$ GST or  $\pi$ GST.
- ii. Calibrators (stock solution).  $\alpha$ GST 4 mg/l: Purified  $\alpha$ GST in stabilizing buffer (200  $\mu$ l), Thiomersal and sodium azide and 5 mg/l or: Purified  $\pi$ GST in 50% (v/v) glycerol (200  $\mu$ l).
- iii. Sample diluent: Protein containing solution (50 ml) and sodium azide.

Positive control: Protein containing solution with added stabilisers (4.5 ml) Thiomersal and sodium azide.

- iv. Conjugate solutions: Anti-  $\alpha$ GST IgG or Anti-  $\pi$ GST IgG conjugated to horseradish peroxidase (1400  $\mu$ l).
- vi. Wash solution: Phosphate buffered saline (PBST 55 ml).
- vii. Substrate: Stabilised liquid tetra methyl benzene (TMB) solution (11 ml).
- viii. Stop solution: 1N sulphuric acid (11 ml).
- ix. Urine stabilizing buffer (10 ml) which contains Thiomersal and sodium azide.

In brief, immediately prior to the analysis, urine samples were diluted (1/2 dilution) by addition of 200  $\mu$ l sample to 200  $\mu$ l sample diluent. Then 100  $\mu$ l of diluted urine, previously prepared calibrators and positive control were placed in the Microassay plate (100  $\mu$ l/well). The assay plate was covered and incubated at room temperature (20-25°C) for 60 minutes with uniform shaking (LUCKHAM, UK Titer-plate shaker was used with speed 2-3).

After exactly 60 minutes, the cover was removed and 250  $\mu$ l of wash solution added to each well and washed 4 times. When this procedure was completed, the plate was firmly tapped against a paper towel to ensure complete removal of wash solution from the wells. Then after addition of 100  $\mu$ l of conjugate solution to each well the assay plate was again covered and incubated at room temperature (20-25°C) for 30 minutes with uniform shaking as described above.

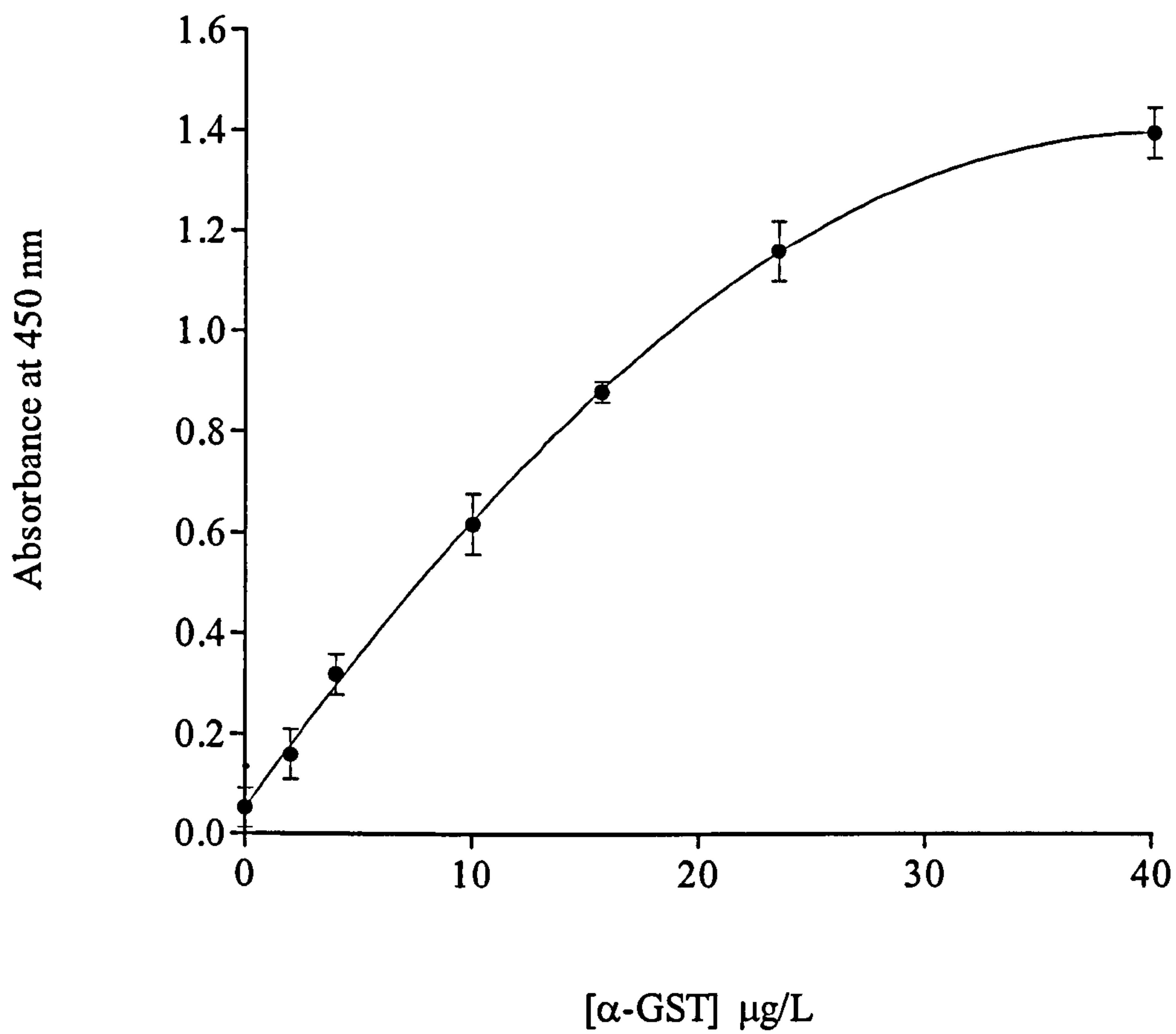
After the exactly 30 minutes, the assay plate was again washed as described earlier. Then substrate 100  $\mu$ l was added to the each well and the assay plate was again incubated at



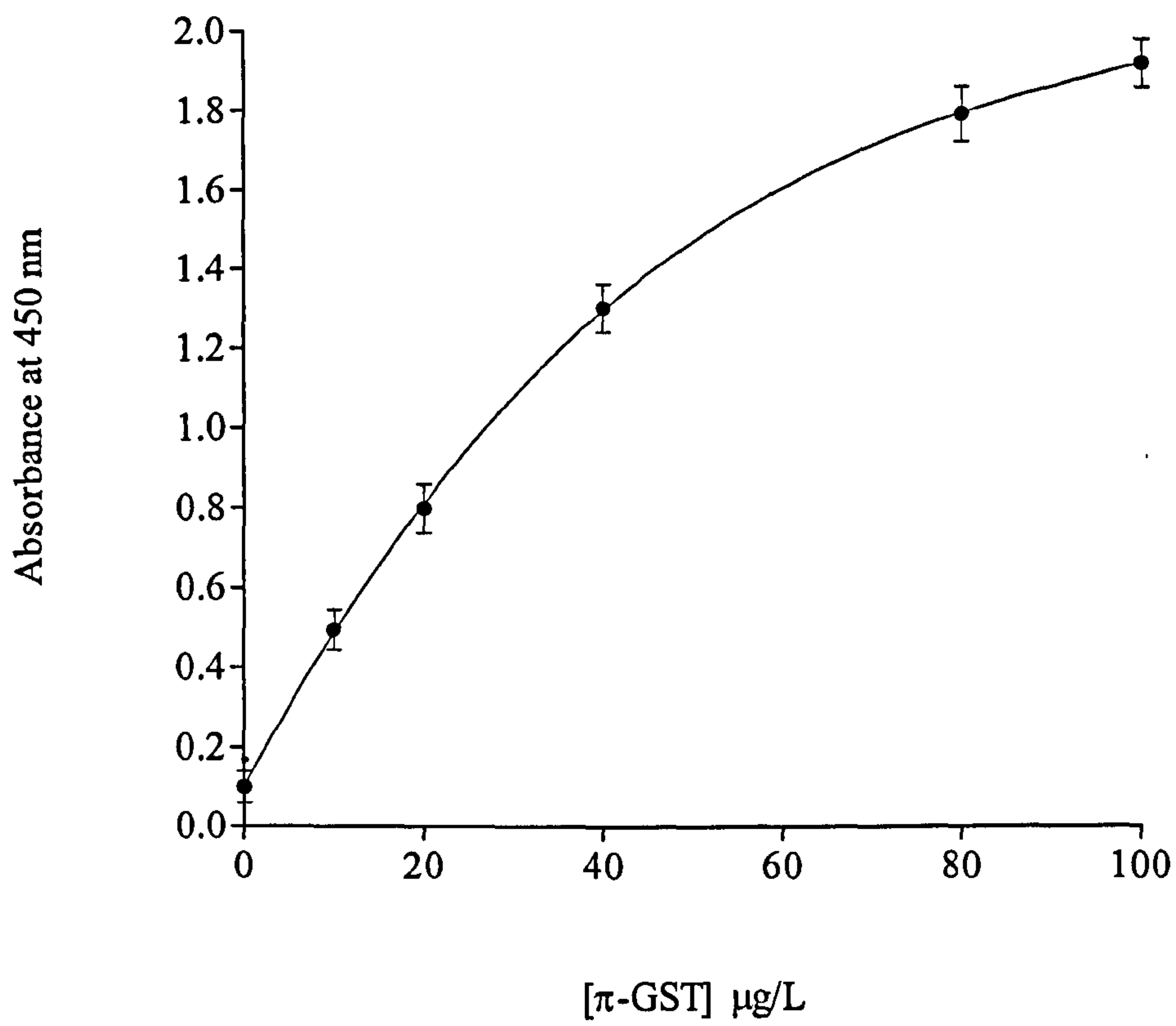
room temperature for 15 minutes. Finally, after the exactly 15 minutes, 100  $\mu$ l of stop solution were added on to each well and immediately readings were taken at 450 nm by using a micro-titer plate reader (Labsystems MS, UK). A typical calibration curve was constructed for both  $\alpha$ GST and  $\pi$ GST (Figure 2.4.9 and 2.4.10) and urine GST activity was expressed as U/mmol urinary creatinine.

## **2.5 Statistical analysis**

Statistical analyses were performed using the GarphadPad Prism Software (1999) Inc, San Diego, Canada.



**Figure 2.4.9** Typical standard curve for the  $\alpha$ GST assay. Results are expressed as mean  $\pm$  SEM for duplicate determinations.



**Figure 2.4.10** Typical standard curve for the  $\pi$ GST assay. Results are expressed as mean  $\pm$  SEM for duplicate determinations.

## **CHAPTER THREE**

### **AN INVESTIGATION OF HYPERGLYCAEMIA AND LIPID METABOLISM IN A DIABETIC POPULATION**



### 3. Introduction

The studies presented in this chapter investigate possible associations between altered metabolic parameters such as glucose, HbA<sub>1c</sub>, fructosamine and lipoproteins in a cohort of both recently diagnosed (group 1) and established (group 2) diabetic subjects. Abnormal glucose metabolism has been shown to be a risk factor for mortality, even in non-diabetic individuals (Feener and King, 1997). As a consequence, a pathological elevation of blood glucose concentration is the cardinal feature of diabetes mellitus. It is well established that hyperglycaemia is associated with altered protein and lipid metabolism resulting in an increased risk of development and progression of both micro and macrovascular complications in diabetes. Furthermore, at the cellular level, the major factors that contribute to and initiate the progression of diabetic microvascular disease are chronic or persistent hyperglycaemia, dyslipidaemia, insulin resistance, various cytokines, vasoactive hormones and free radical mediated oxidative stress (Feener and King, 1997; Baynes and Thorpe, 1999; West, 2000). However, the relative contribution of these factors to the development and progression of diabetic microvascular complications such as diabetic nephropathy, retinopathy and neuropathy depends on the specific vascular tissue affected and on the particular stage of the disease (Baynes, 1991; Giugliano *et al*, 1996; Feener and King, 1997; Baynes and Thorpe, 1999; West, 2000). Consequently, several theories have emerged to describe the adverse effects of hyperglycaemia on diabetic specific complications and these include glucose autooxidation, non-enzymatic glycation of both extracellular and intercellular proteins, metabolism of glucose and fructose via the polyol pathway and altered lipid metabolism

associated with the generation of free radicals thereby enhancing oxidative stress that may ultimately leading to the progression and the development of so-called diabetic specific complications (Baynes, 1991; Brownlee, 1995; Schwartz, 1992; DCCT, 1993; UKPDS,1998).

### **3.1 Materials and methods**

The study groups, materials and methods are as described in Chapter 2, Sections 2.2.1 to 2.2.7.

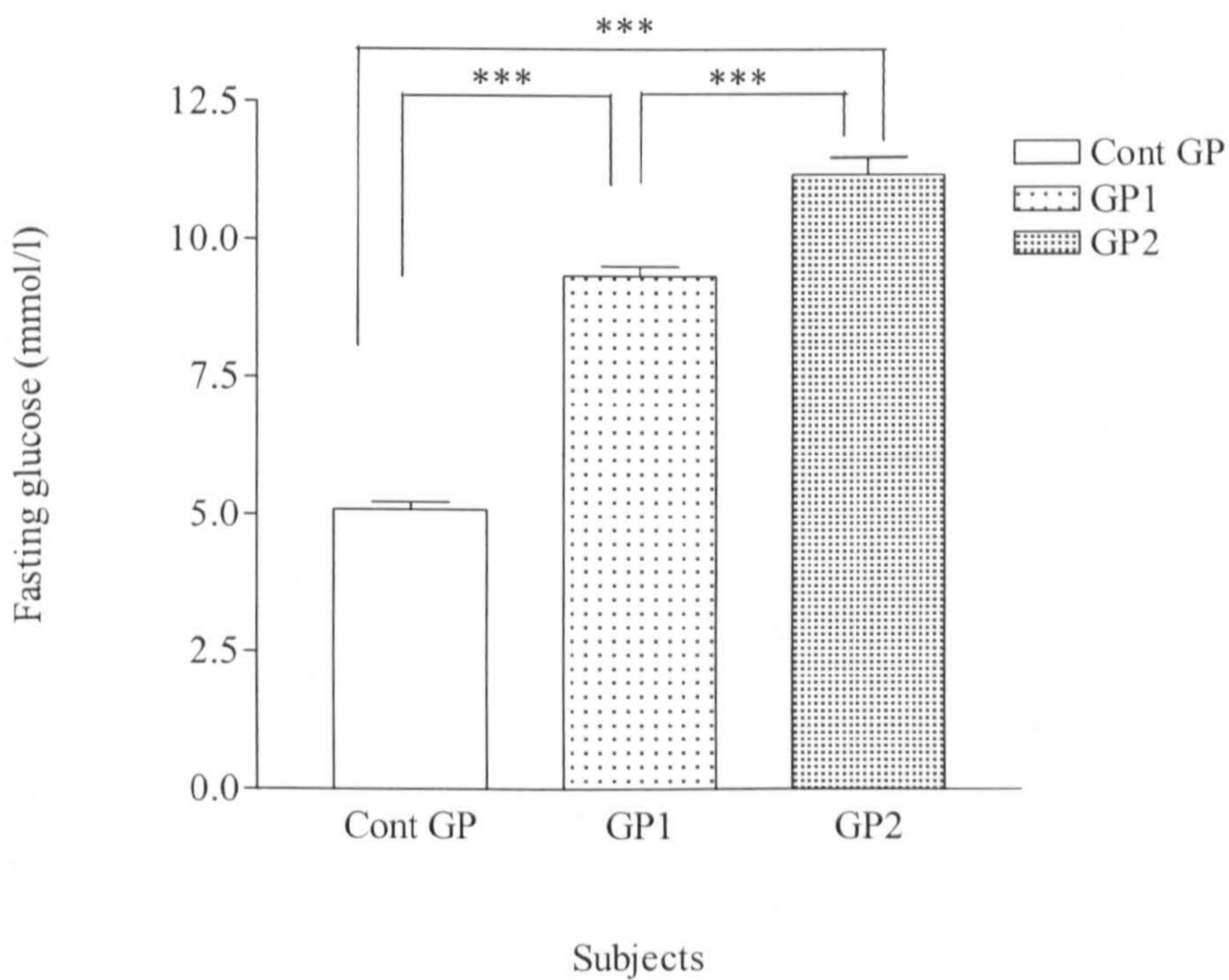
### **3.2 Results**

#### **3.2.1 Fasting plasma glucose concentrations (Figure 3.2.1)**

Fasting plasma glucose concentrations (mmol/l) were significantly increased in both recently diagnosed diabetic group (GP1) and established diabetic group (GP2) compared with non-diabetic control group values (Cont GP) (Cont GP vs GP1 vs GP2,  $P < 0.001$ ). Higher plasma glucose concentrations were observed in the established diabetic group (GP2) compared with both the recently diagnosed diabetic group (GP1) and control group subjects (both  $P < 0.001$ ). Fasting glucose concentrations in the control group were within the laboratory reference intervals (4.2-6.1 mmol/l).

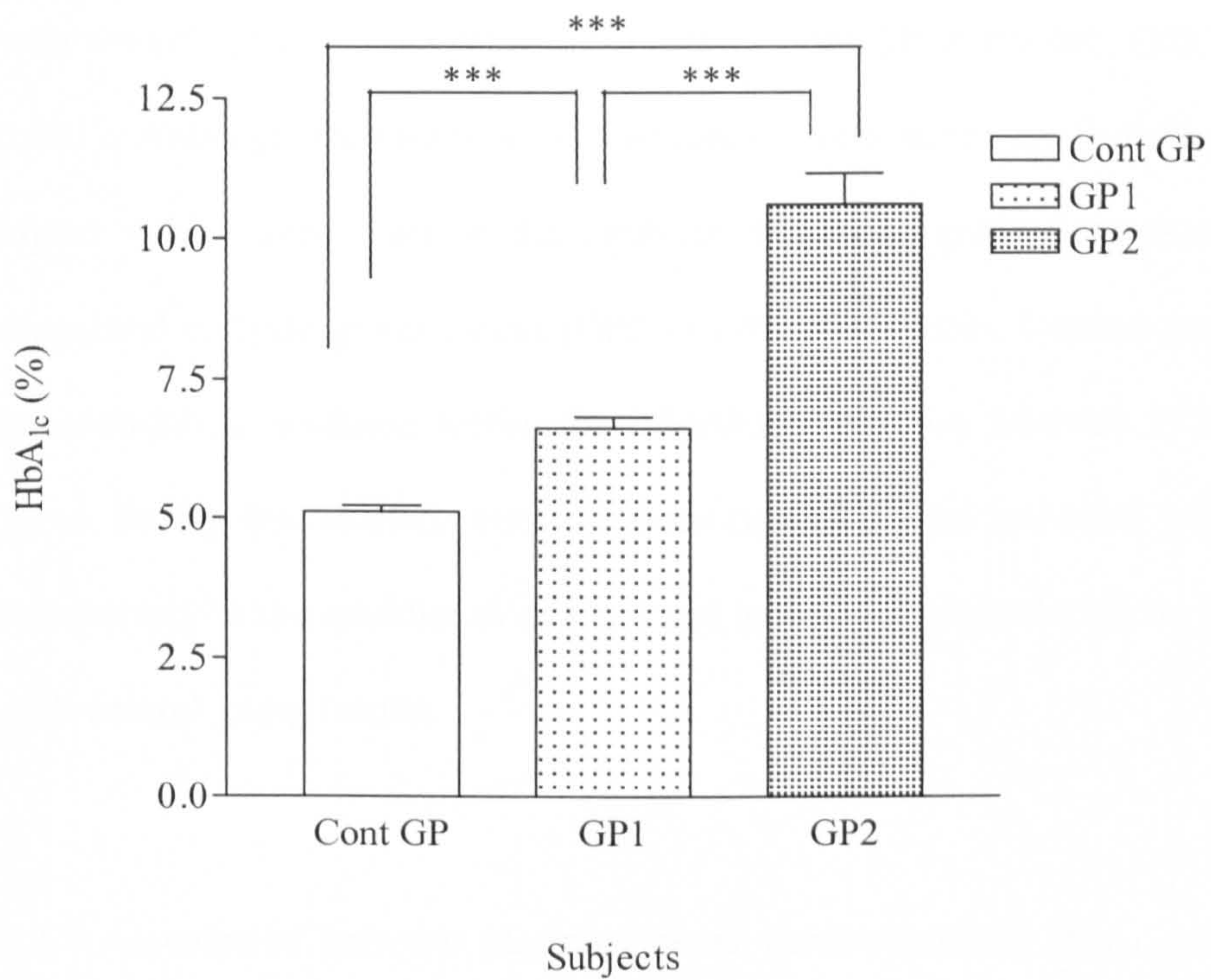
#### **3.2.2 Glycated haemoglobin (HbA<sub>1c</sub>) levels (Figure 3.2.2)**

HbA<sub>1c</sub> (%) values were significantly increased in both the established diabetic (GP2) and recently diagnosed diabetic (GP1) groups compared with control group (Cont GP) values (Cont GP vs GP1,  $P < 0.001$ , Cont GP vs GP2,  $P < 0.001$ ). HbA<sub>1c</sub> values were significantly higher in the established diabetic group than those observed in the recently diagnosed diabetic group values (GP1vs GP2,  $P < 0.001$ ). In the control group, HbA<sub>1c</sub> levels were within the laboratory reference range (4.4 %-5.7 %).



**Figure 3.2.1** Fasting plasma glucose concentrations (mmol/l) in control group (Cont GP), recently diagnosed diabetic group or group 1 (GP1) and established diabetic group or group 2 (GP2) subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by Bonferroni's multiple comparison test. (\*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ ).





**Figure 3.2.2** HbA<sub>1c</sub> (%) levels in control group (Cont GP), GP1 and GP2 subjects. Results (mean ± SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ ).

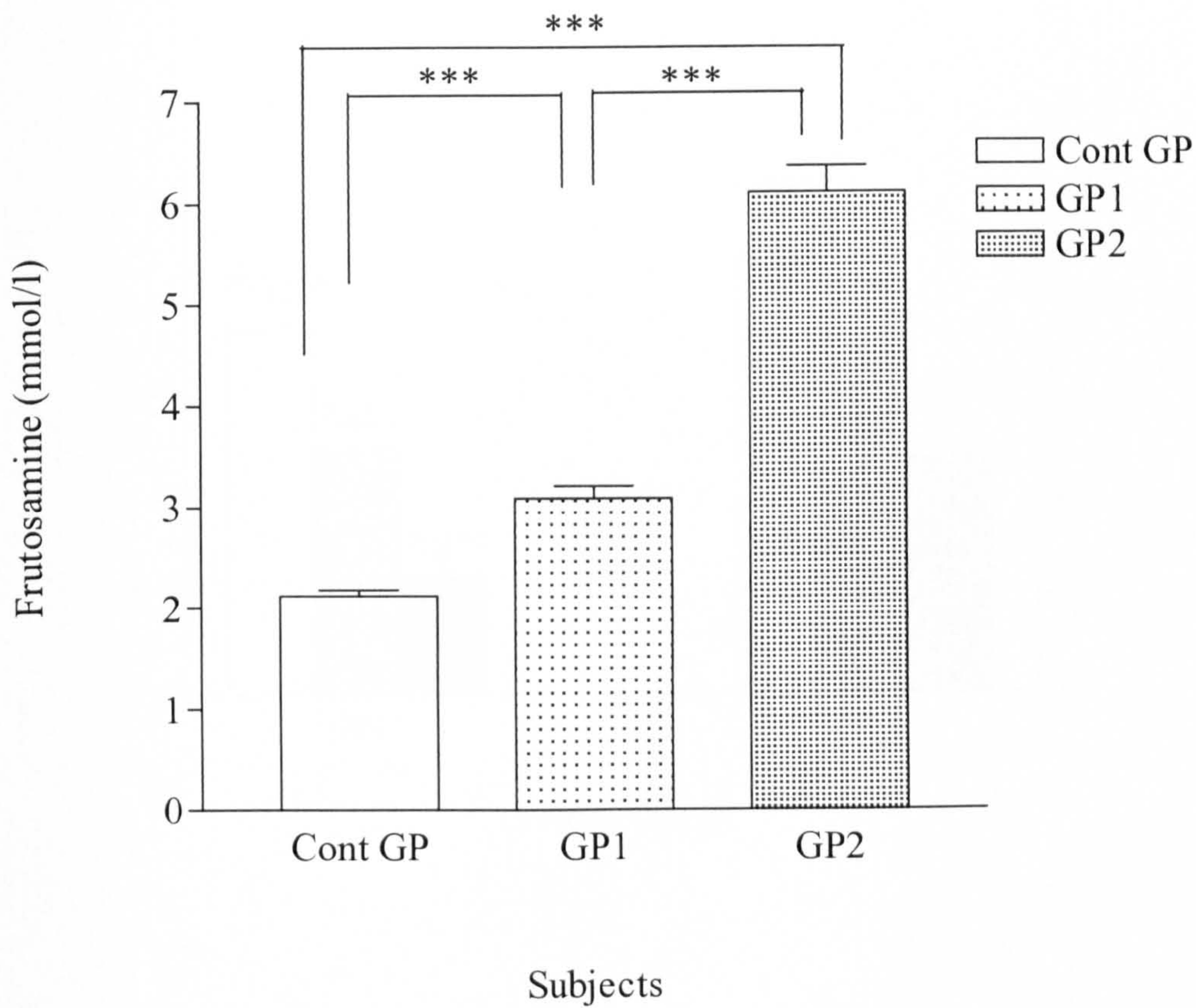


### **3.2.3 Fructosamine concentrations (Figure 3.2.3)**

Fasting fructosamine concentrations (mmol/l) were also significantly increased in both the recently diagnosed diabetic (GP1) and established diabetic (GP2) groups compared with control group (Cont GP) results (GP1 vs Cont GP,  $P < 0.001$ , GP2 vs Cont GP,  $P < 0.001$ ). Although fructosamine concentrations were increased in both diabetic groups, higher values were noted in the established diabetic group compared to the recently diagnosed diabetic group values (GP2 vs GP1,  $P < 0.001$ ). Control group fructosamine concentrations remained within the laboratory reference intervals (1.87-2.87 mmol/l). Mean fasting fructosamine concentrations (mmol/l) were increased 3-fold and 1.5-fold respectively in the established diabetic and recently diagnosed diabetic groups compared with control group results.

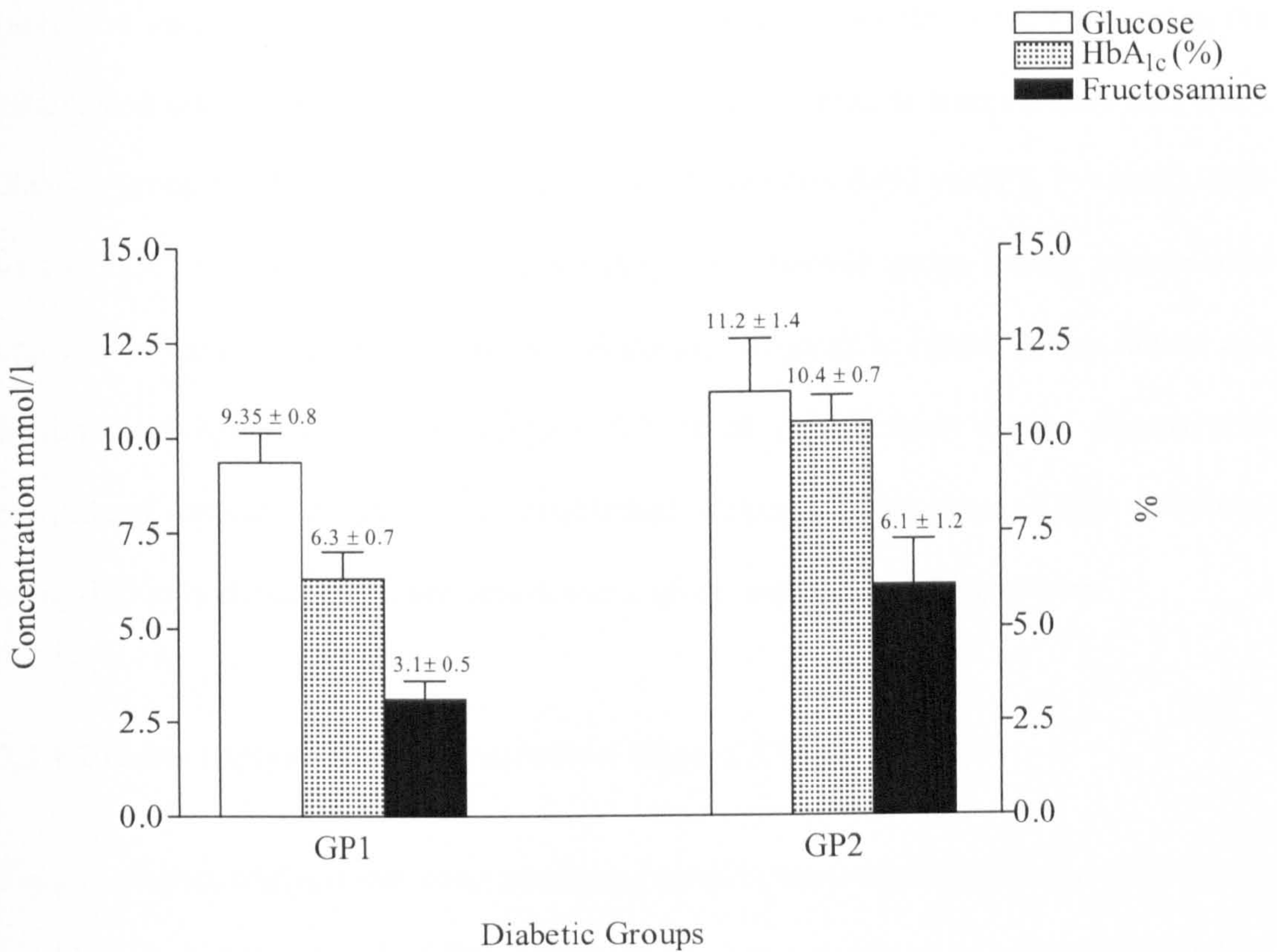
### **3.2.4 Association between plasma glucose concentrations, HbA<sub>1c</sub> and fructosamine levels in both diabetic groups (Figure 3.2.4)**

In the established diabetic group (GP2), mean fasting blood glucose concentrations, HbA<sub>1c</sub> and fructosamine values were significantly increased compared to recently diagnosed diabetic group (GP1) values (glucose: GP2 vs GP1,  $P < 0.001$ , fructosamine: GP2 vs GP1,  $P < 0.001$ , HbA<sub>1c</sub>: GP2 vs GP1,  $P < 0.001$ ). There was however, no significant correlation noted between the above parameters in these two groups.



**Figure 3.2.3** Fasting fructosamine (mmol/l) levels in control group (Cont GP), GP1 and GP2 subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ ).





**Figure 3.2.4** Fasting glucose concentrations, HbA<sub>1c</sub> levels and fructosamine concentrations in the recently diagnosed diabetic group (GP1) and established diabetic group subjects (GP2). Results are expressed as the mean ± (SEM). Numerical values are given above the error bars.



### **3.2.5 Plasma total cholesterol concentrations (Figure 3.2.5)**

Increased fasting plasma total cholesterol concentrations (mmol/l) were observed in the established diabetic group (GP2) compared to those observed in both recently diagnosed diabetic group (GP1) and control group (Cont GP) subjects (GP2 vs GP1,  $P < 0.001$ , GP2 vs Cont GP,  $P < 0.001$ ). In the recently diagnosed diabetic group fasting plasma total cholesterol values were not significantly different compared to control group values, and within the laboratory reference range ( $<5.2$  mmol/l). The mean fasting plasma total cholesterol concentrations in the established diabetic group were 2-fold increased compared to both recently diagnosed diabetic group and control group values.

### **3.2.6 Plasma triglycerides concentrations (Figure 3.2.6)**

Fasting plasma triglycerides concentrations (mmol/l) were significantly increased in the established diabetic group (GP2) compared to those observed in recently diagnosed diabetic group (GP1) and control group (Cont GP) subjects (GP2 vs GP1,  $P < 0.001$ , GP2 vs Cont GP,  $P < 0.001$ ). Recently diagnosed diabetic group and control group plasma triglycerides were similar and within the reference range ( $<2.3$  mmol/l). The mean fasting plasma triglycerides concentrations were increased 2-fold in the established diabetic group compared to both recently diagnosed diabetic group and control group values.



### **3.2.7 Plasma HDL cholesterol concentrations (Figure 3.2.7)**

Plasma HDL cholesterol concentrations (mmol/l) were significantly decreased in the established diabetic group (GP2) compared with both recently diagnosed diabetic group (GP1) and control group (Cont GP) values. (GP2 vs GP1,  $P < 0.001$ , GP2 vs Cont GP,  $P < 0.001$ ). In the recently diagnosed diabetic group plasma HDL cholesterol values were similar to control group values and within the laboratory reference range ( $>0.9$  mmol/l). The mean plasma HDL cholesterol concentration values were reduced 3-fold in the established diabetic group compared to both recently diagnosed diabetic group and control group values.

### **3.2.8 Plasma LDL cholesterol concentrations (Figure 3.2.8)**

Increased plasma LDL cholesterol concentrations (mmol/l) were also noted in the established diabetic group (GP2) compared with both recently diagnosed diabetic group (GP1) and control group (Cont GP) values (GP2 vs GP1,  $P < 0.001$ , GP2 vs Cont GP,  $P < 0.01$ ). Plasma LDL cholesterol concentrations in the recently diagnosed diabetic group were similar to control group values and within the laboratory reference intervals (2.6-3.9 mmol/l). The mean plasma LDL cholesterol concentration values were around 3-fold increased in the established diabetic group compared to both recently diagnosed diabetic group and control group values.

### **3.2.9 Relation of plasma total cholesterol triglycerides, HDL and LDL concentrations in both diabetic groups (Figure 3.2.9)**

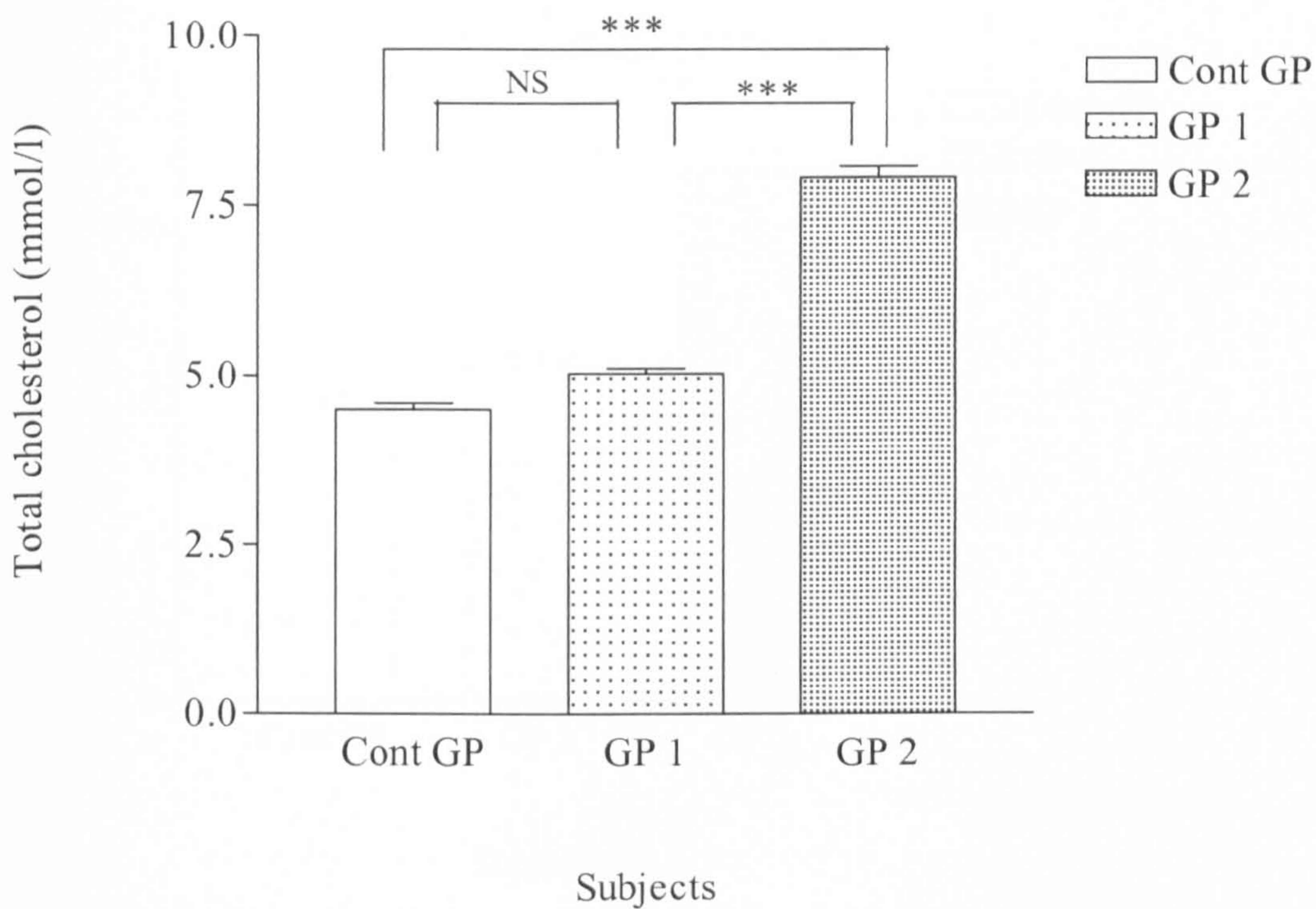
In the established diabetic group (GP2), the mean concentrations of plasma total cholesterol triglycerides, HDL and LDL were significantly increased compared to those in the recently diagnosed diabetic group (GP1) (cholesterol: GP2 vs GP1,  $P < 0.001$ , triglycerides: GP2 vs GP1,  $P < 0.001$ , HDL: GP2 vs GP1,  $P < 0.001$ , LDL: GP2 vs GP1,  $P < 0.001$ ). However, significant correlations were not observed with these parameters in these two groups.

### **3.2.10 LDL:HDL ratio (Figure 3.2.10)**

LDL:HDL ratio were significantly increased in the established diabetic group (GP2) compared to both recently diagnosed diabetic group (GP1) (GP2 vs GP1,  $P < 0.001$ ) and control group (Cont GP) (GP2 vs Cont GP,  $P < 0.01$ ) values. In the recently diagnosed diabetic group LDL:HDL ratio was similar when compared to control group values.

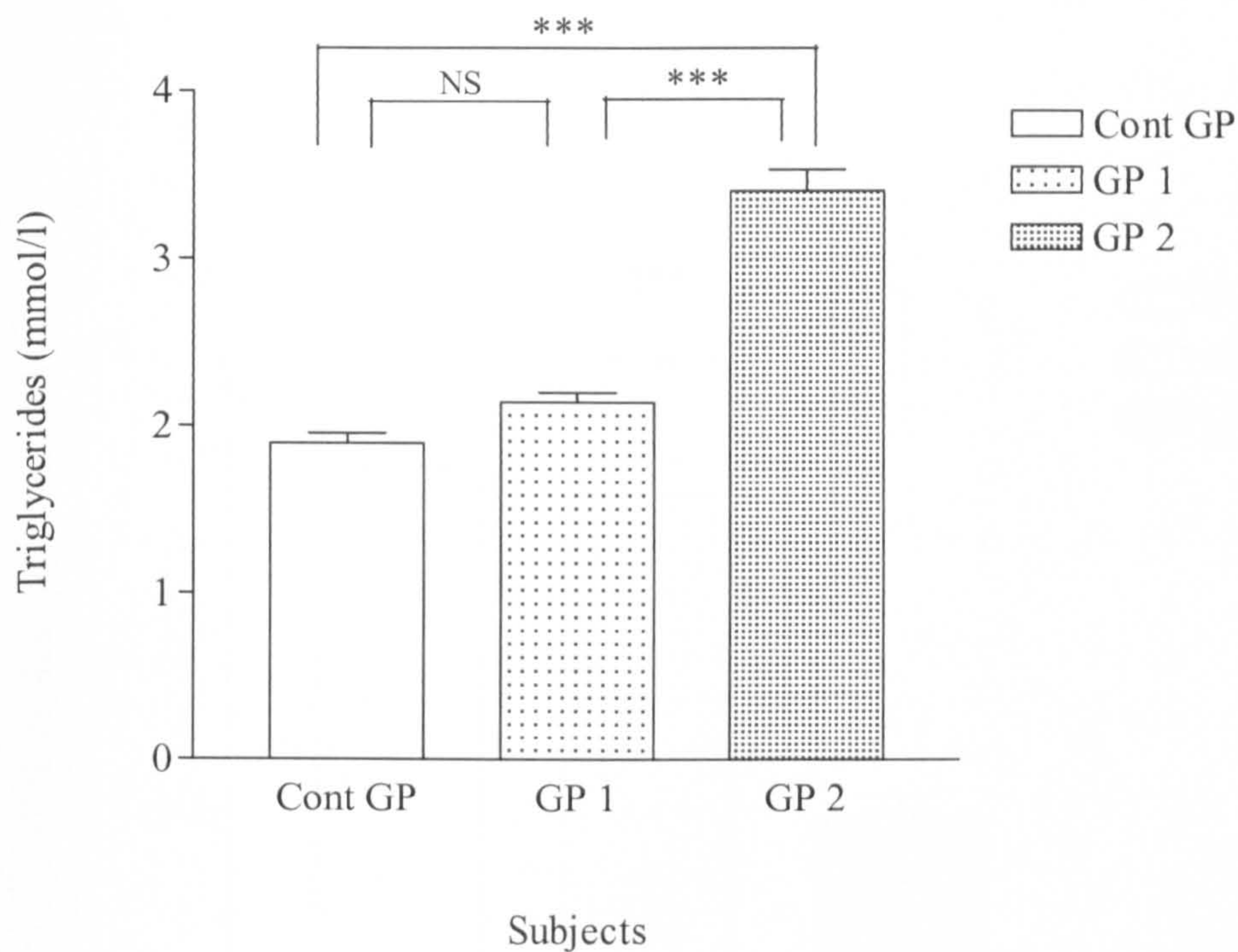
### **3.2.11 (LDL+TG):HDL ratio (Figure 3.2.11)**

(LDL+TG):HDL ratio were also significantly increased in the established diabetic group (GP2) compared to both recently diagnosed diabetic group (GP1) (GP2 vs GP1,  $P < 0.001$ ) and control group (Cont GP) (GP2 vs Cont GP,  $P < 0.01$ ) values. In the recently diagnosed diabetic group (LDL+TG):HDL ratio was similar when compared to control group values.

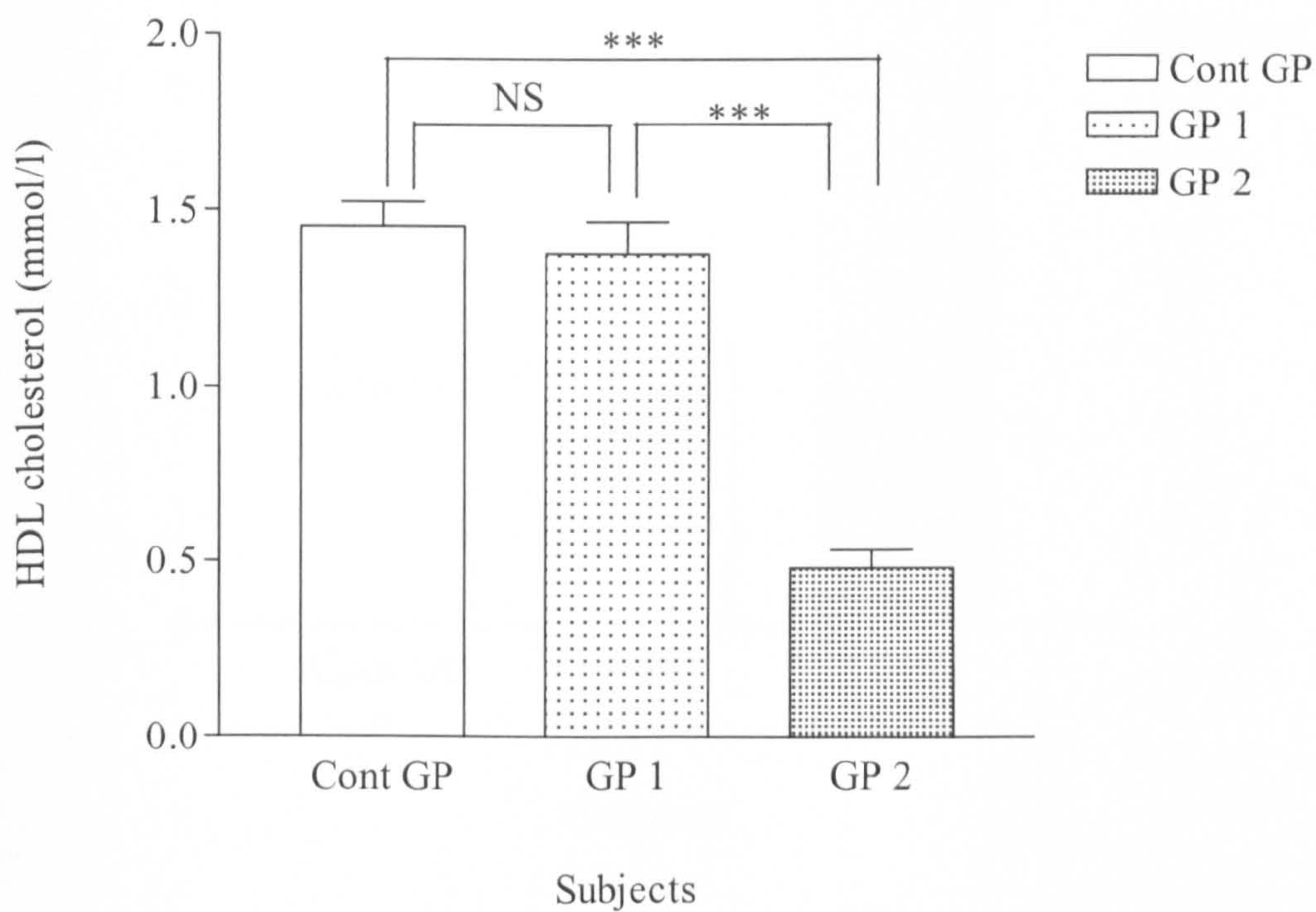


**Figure 3.2.5** Fasting plasma total cholesterol concentrations (mmol/l) in control group (Cont GP), GP1 and GP2 subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).



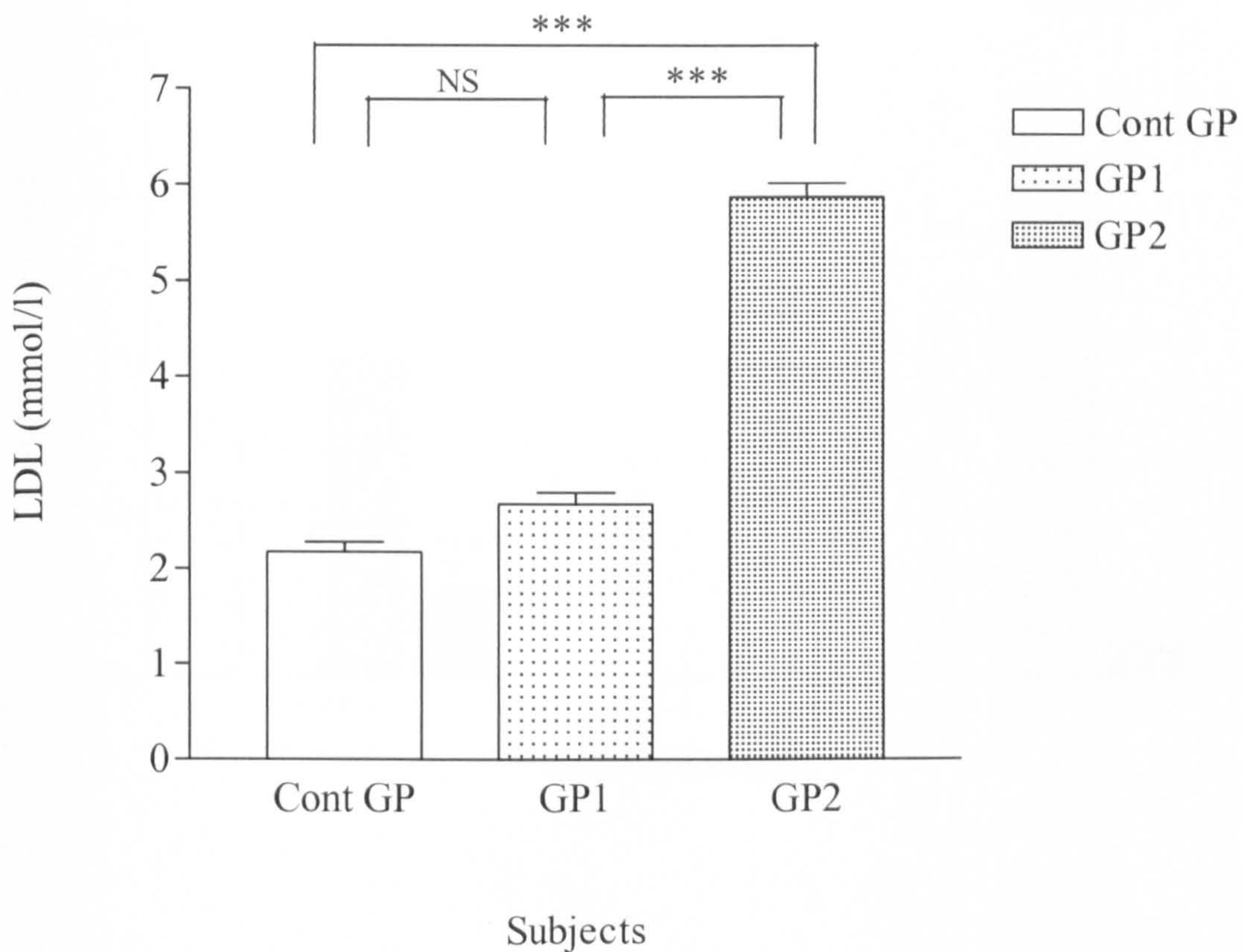


**Figure 3.2.6** Fasting plasma triglycerides concentrations (mmol/l) in control group (Cont GP), GP1 and GP2 subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).



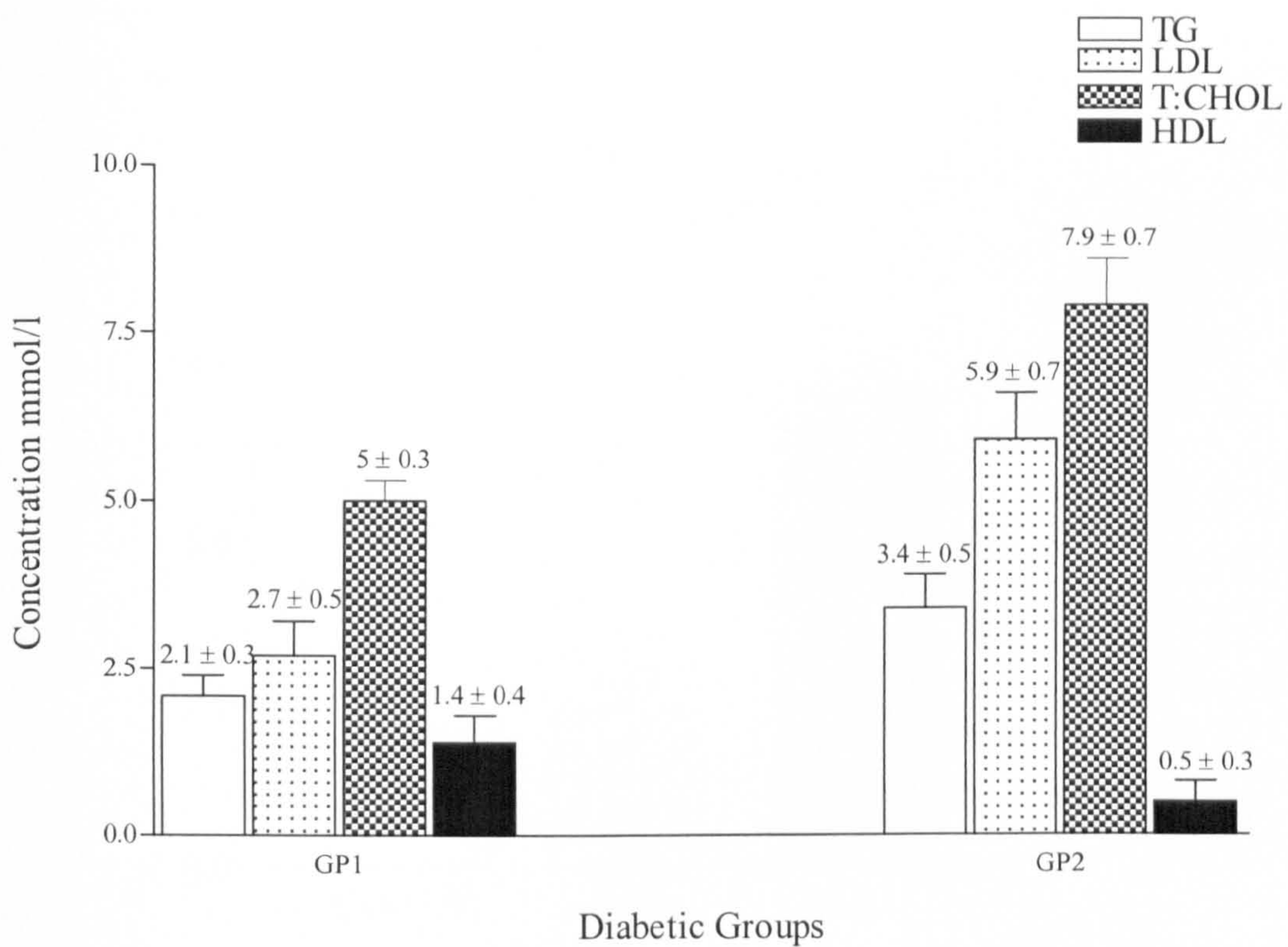
**Figure 3.2.7** Fasting plasma HDL concentrations (mmol/l) in control group (Cont GP), GP1 and GP2 subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).



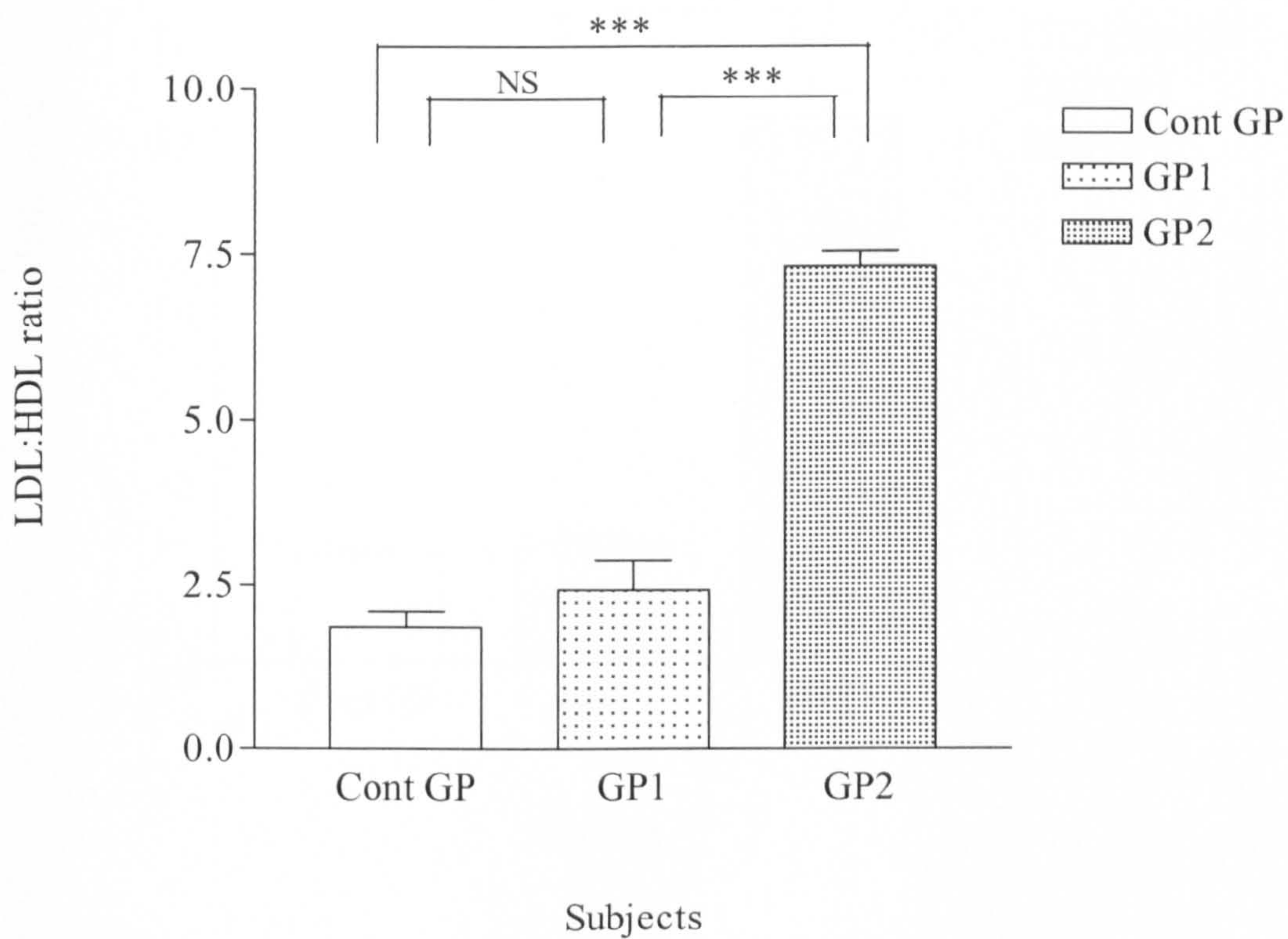


**Figure 3.2.8** Plasma LDL concentrations (mmol/l) control group (Cont GP), GP1 and GP2 subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).





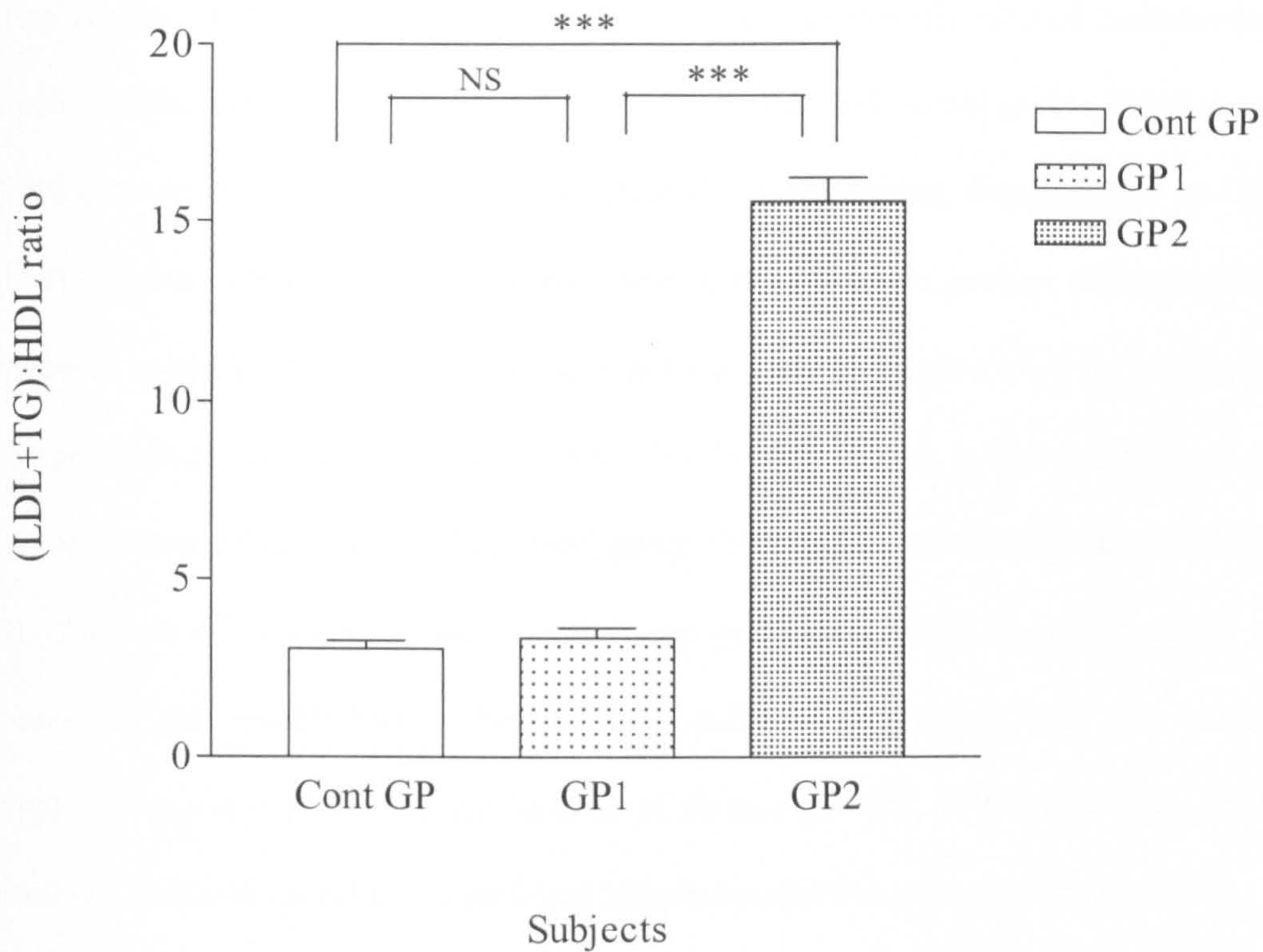
**Figure 3.2.9.** Plasma total cholesterol (T:CHO), triglycerides (TG), LDL and HDL concentrations in the recently diagnosed diabetic group (GP1) and established diabetic group (GP2). Results are expressed as the mean ± (SEM). Numerical values are given above the error bars.



**Figure 3.2.10** LDL:HDL ratio in control group (Cont GP), GP1 and GP2 subjects.

Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).





**Figure 3.2.11** (LDL+TG):HDL ratio in control group (Cont GP), GP1 and GP2 subjects.

Results (mean ± SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).



### 3.3 Discussion

The results of the studies presented here reveal significantly altered carbohydrate and lipid metabolism in both the recently diagnosed and established diabetic subjects. Furthermore, increased fasting plasma glucose concentrations, fructosamine and glycated haemoglobin (HbA<sub>1c</sub>) levels were observed in both diabetic groups compared with non diabetic control groups. However, and not surprisingly, higher fasting plasma glucose concentrations, fructosamine and HbA<sub>1c</sub> levels were noted in the established diabetic group compared to recently diagnosed group subjects. As previously described (Chapter 2), duration of the diabetes was less than two years in recently diagnosed group and 6-8 years in the established diabetic group subjects suggesting that the severity of hyperglycaemia is related to the duration of disease (DCCT, 1993). However, correlation analysis failed to reveal any significant association between these parameters.

Distribution of fasting plasma glucose concentration within the general population provides valuable information for comparison with those subjects with diabetes. Many studies have demonstrated that increased fructosamine and HbA<sub>1c</sub> levels in both type 1 and type 2 uncontrolled diabetes directly correlate with fasting plasma glucose concentrations (DCCT, 1993; DCCT 1995; Goldstein *et al*, 1995; UKPDS, 1998; ADA, 2002). Nevertheless, in the preset study a significant direct correlation between fasting glucose concentrations and HbA<sub>1c</sub> levels was not observed. This of course may be due to the relatively small sample populations (Goldstein *et al*, 1995) when compared to the large scale clinical prospective studies. The formation of HbA<sub>1c</sub> occurs over the average

life span of erythrocytes (approximately 120 days) and depends on the timed average glucose concentration over this period and measurement of both glycated haemoglobin and proteins in subjects with diabetes is an established procedure for evaluating long-term glycaemic control (Goldstein *et al*, 1995; ADA, 2002). Consequently, the results of the present study are consistent with these observations and confirm that prolonged exposure to abnormal glucose concentration (i.e. persistent hyperglycaemia resulting in poor glycaemic control) is associated with abnormal elevation of glycated proteins and albumin. Furthermore, many prospective clinical studies have demonstrated that intensified glycaemic control was associated with a reduced risk for the development and progression of diabetic specific microvascular complications such as nephropathy, retinopathy and neuropathy. (DCCT, 1993; Ohkubo *et al*, 1995; Stratton *et al*, 2000). Recently, the United Kingdom prospective diabetes study (UKPDS, 1998) group demonstrated that maintaining blood glucose concentrations as close to normal as possible prevented and retarded the development of diabetic nephropathy.

Hyperglycaemia appears to damage tissue by acute but reversible changes in metabolism. In the setting of hyperglycaemia, no-enzymatic glycation results from the interaction of aldoses, such as glucose, with free amino groups on polypeptides or lipids. Formation of early glycation end products, such as Schiff bases and Amadori products, the best known of which is haemoglobin A<sub>1c</sub>, is reversible. Further molecular rearrangements, often involving oxidation, eventually result in the formation of AGEs (Brownlee, 1995). The rate of formation of AGEs exceeds that predicted by first order kinetics and this implies that, over time, even modest hyperglycaemia can result in a significant accumulation of



AGEs on long-lived macromolecules. This is well established for certain long-lived proteins such as those of the ocular lens. For example, the progressive post-translational modification on lens crystallins by glucose-derived AGEs explains the premature lenticular browning and cumulative cross-linking occurring during the course of diabetes (Brownlee, 1984). Immunohistochemical studies of kidney from normal and diabetic animals have suggested that GBM, mesangium, podocytes, and renal tubular cells accumulate high concentrations of AGEs that may ultimately be associated with diabetic nephropathy (Vlassara, 1995). Recent studies have demonstrated that AGEs appears to stimulate the generation of free radicals, thereby enhancing oxidative stress in the diabetic state (Yan *et al*, 1994; Schmidt *et al*, 1996; Vlassara, 1996).

A complications linkage between hyperglycaemia, hyperlipidaemia, and atherosclerosis in the pathogenesis of diabetic complications is highly probable. Several studies have suggested that hyperglycaemia itself, and closely associated risk factors including insulin resistance, obesity, hypertension and lipid abnormalities are important predisposing factors. Diabetic subjects with altered lipid metabolism are at high risk of vascular disease, particularly those with symptoms of diabetic nephropathy (Friedman, 1999). Diabetic dyslipidaemia is often called atherogenic dyslipidaemia and is characterised by lipoprotein abnormalities including elevated total cholesterol, triglycerides and LDL, and low HDL concentrations. Increased total cholesterol, triglycerides and LDL, and decreased HDL concentrations were noted in the established diabetic group compared to both recently diagnosed diabetic group and control group subjects (Figure 3.2.6, 3.2.7, 3.2.8, 3.2.9) in the present study. In addition, increased LDL:HDL and (LDL+TG):HDL



ratios (Figure 3.2.10, 3.2.11) were observed in the established diabetic group when compared to recently diagnosed diabetic group. Although increased fasting glucose concentrations, fructosamine and HbA<sub>1c</sub> levels were associated with increased total cholesterol, triglycerides and LDL, and decreased HDL concentrations in established diabetic group subjects, no significant correlations could be demonstrated.

The degree of glycaemic control is an important determinant of serum lipoprotein concentrations in diabetes mellitus (Taskinen 1987). Consequently, serum cholesterol and triglyceride HDL and LDL concentrations provide a first impression in diabetic subjects of altered lipid metabolism, and provide valuable information for further diagnostic interpretation, therapy, and monitoring for hyperlipidaemia in diabetes (Chait and Haffner, 2001). The results of the present study are consistent with these observations and confirmed that poor metabolic control associated with altered lipid metabolism was present in the established diabetic group subjects. Furthermore, in diabetes, moderate hypertriglyceridaemia associated with a reduced concentration of HDL cholesterol is a common observations (Betteridge, 1994), again results similar to those observed in the present study. The Framingham study revealed that the prevalence of hypertriglyceridaemia in subjects with type 2 diabetes was significantly increased compared with non-diabetic control subjects values and significantly correlated with poor glycaemic control (Miettinen *et al*, 1998). Despite adequate glycaemic control, mild hypertriglyceridaemia and decreased HDL concentrations often persist in subjects with type 2 diabetes. Diabetic hypertriglyceridaemia is based on enhanced hepatic very low-density lipoprotein (VLDL) secretion and diminished VLDL and chylomicron clearance

(Koschinsky and Gries, 1992). As a consequence of their cholesterol content, an increase in triglyceride rich lipoproteins leads to an elevation in total serum cholesterol concentration (Betteridge, 1997). When glycaemic control is poor, total serum cholesterol concentrations in diabetes can be significantly enhanced due to the additional accumulation of LDL (Taskinen *et al*, 1996) as observed in the established diabetic group in the present study. Moreover, diabetic subjects with good or reasonable glycaemic control still tend to demonstrate increased LDL cholesterol concentrations. Nevertheless, LDL catabolism is impaired in subjects with moderately severe diabetes, resulting in an increased proportion of triglycerides. The increase in the concentration of small, dense LDL in serum, their glycation and oxidative modification (Brownlee *et al*, 1984) contributes to the increase of total serum LDL in poorly controlled diabetic subjects. The size and density of LDL is considerably influenced by changes in triglycerides content. Under conditions of poor glycaemic control, LDL in type 2 diabetic subjects is characterised by an elevated proportion of triglycerides, mostly at the expense of cholesterol (Kramer-Guth *et al*, 1996; Kramer-Guth *et al*, 1997). Alterations in the lipid composition or size of LDL particles have consequences for their binding to lipoprotein receptors. Triglyceride-rich and small dense LDL demonstrate a reduced cellular uptake via the LDL receptor, leading to accumulation of LDL in the vascular system which is associated with the development of diabetic complications (Kramer-Guth *et al*, 1996).

Dyslipidaemic changes are evident in diabetic subjects and are characterised by increased concentrations of LDL, which significantly predispose these subjects to atherosclerosis, with a subsequent increased risk of coronary heart and kidney diseases. (Bucala *et al*,



1993). Advanced glycation of the lipid component of LDL occurs concomitantly with LDL oxidation *in vivo* (Bucala *et al*, 1993). During glycation, fatty acid residues can be oxidized independently of transition metals resulting in the generation of free radicals (Bucala *et al*, 1993; Bucala *et al*, 1994). Furthermore, it has been suggested that lipid-advanced glycation and oxidation increase during the persistent hyperglycaemia of diabetes, further support the concept that AGE oxidation plays an important and probably primary role in initiating lipid oxidation *in vivo* and thereby enhance free radical formation (Bucala *et al*, 1992; Bucala *et al*, 1993).

In contrast, the observation in the present study is consistent with evidence from other studies that suggests the persistent hyperglycaemia can exert pathological effects in diabetes. As a consequence of increased formation of non-enzymatic glycation of protein and altered lipids metabolism, this may result in an increase of free radical production thereby enhancing oxidative stress that may eventually lead to progression and the development of diabetic specific complications. Evidence suggests that in subjects with diabetes increased free radical production associated with increased lipid peroxidation and depletion of antioxidant mechanisms results in enhanced oxidative stress that eventually lead to enhanced development/progression of diabetic nephropathy. However, the next chapter addresses the association between lipid peroxidation and antioxidants in both recently diagnosed diabetic and established diabetic subjects.



## **CHAPTER FOUR**

### **AN INVESTIGATION OF LIPID PEROXIDATION AND ANTIOXIDANT BIOCHEMICAL MARKERS IN A DIABETIC POPULATION**

#### 4. Introduction

Free radical mediated events in biochemical processes play a significant pathophysiological role in disease process, implicated in a number of disorders including diabetes and its complications (Oberley, 1988; Baynes, 1991; Packer, 1993; Baynes, 1996; West, 2000). The intracellular or extracellular conditions that result in either the chemical or the metabolic generation of reactive species is known as oxidative stress. Generally, metabolic activity, in conjunction with the structural organisation of the cell, is able to control or prevent the adverse effects of oxidative stress. The susceptibility of a given organ or organ system to oxidative stress is a function of the overall balance between the factors that exert oxidative stress and those that exhibit antioxidant capability. Oxidative damage can, therefore, be described as a consequence of excessive oxidative stress via formation of free radicals or insufficient antioxidant potential. Consequently, oxidative stress is defined as a disturbance in the balance between the production of free radicals and antioxidant defences, which may eventually lead to tissue injury (Halliwell, 1994; Betteridge, 2000). Increased oxidative stress may result from the overproduction of precursors of reactive oxygen radical and/or a decreased efficiency of inhibitory and scavenger systems. Oxidative stress then may be amplified and propagated by an autocatalytic cycle of metabolic stress, tissue damage, and cell death, leading to a simultaneous increase in free radical production and compromised inhibitory and scavenger mechanisms, which further exacerbate the severity of the oxidative stress (Baynes, 1991; Baynes and Thrope, 1999). The potential contribution of increased oxidative stress to the development and progression of diabetic complication in general,

and diabetic nephropathy in particular, may be associated with a number of biochemical pathways. It is now well recognised that hyperglycaemia is the major pathophysiological contributor to the development of diabetic complications. Increased generation of reactive oxygen metabolites, such as superoxide anions and hydrogen peroxide, has been demonstrated to occur in diabetes in response to hyperglycaemia (Baynes and Thrope, 1999).

Baynes, (1991) suggested that oxidative stress might be a common pathway linking diverse mechanisms for the pathogenesis of diabetic complications. Mechanisms that contribute to increased oxidative stress in diabetes may include not only increased non-enzymatic glycosylation and autooxidative glycosylation, but also metabolic stress resulting from changes in energy metabolism, alteration in sorbitol pathway activity, changes in inflammatory mediators, the status of antioxidant defence systems, and localized tissue damage resulting from hypoxia and ischaemic-reperfusion injury. However, in diabetes, oxidative stress is mainly caused by both an increased production of plasma free radicals associated with an increased lipid peroxidation and a sharp reduction in antioxidant defences (Jain *et al*, 1989; Griesmacher, 1994; Giugliano *et al*, 1996). Consequently, the main aims of the present study is to evaluate the association between lipid peroxidation and antioxidant activities in recently diagnosed and established diabetic subjects.



## **4.1 Materials and methods**

Investigated study groups, materials and methods are as described in Chapter 2 Sections 2.3.1 to 2.3.5

## **4.2 Results**

### **4.2.1 Lipid peroxidation and antioxidant markers in diabetes mellitus**

An initiate of lipid preoxidation was assessed by using the TBARS (MDA) assay as described in Section 2.3.1 and antioxidants such as total antioxidant status (Section 2.3.3),  $\alpha$ -tocopherol (vitamin E) (Section 2.3.2), glutathione peroxidase (Section 2.3.4) and selenium (Section 2.3.5) were analysed by the standard methods as described in Chapter 2.

### **4.2.2 Urinary Thiobarbituric Acid Reactive Substances (TBARS) (Figure 4.2.2)**

Increased urinary TBARS levels ( $\mu\text{mol}/\text{mmol}$  creatinine) were observed in both diabetic groups [recently diagnosed diabetic group (GP1) and established diabetic group (GP2)] compared with the nondiabetic control group (Cont GP) subjects (Cont GP vs GP1 vs GP 2,  $P < 0.001$ ). The mean TBARS levels were increased 3-fold and 2-fold respectively in the established diabetic and recently diagnosed diabetic groups compared with control group (Cont GP).

#### **4.2.3 Association between TBARS and HbA<sub>1c</sub> levels in the established diabetic group (Figure 4.2.3)**

In the established diabetic group (GP2) a significant correlation between urinary TBARS ( $\mu\text{mol/l}$ ) and HbA<sub>1c</sub> (%) was observed ( $n = 20$ ,  $y = 3.6802x - 2.1447$ ,  $r^2 = 0.894$ ,  $P < 0.0001$ ).

#### **4.2.4 Plasma total antioxidant status (Figure 4.2.4)**

Plasma total antioxidant status ( $\text{mmol/l}$ ) was significantly decreased in the established diabetic group (GP2) compared to recently diagnosed diabetic (GP2 vs GP1,  $p < 0.001$ ) and control groups (GP2 vs Cont GP,  $p < 0.001$ ) values. However, in the recently diagnosed diabetic and control groups plasma total antioxidant status levels were not significantly different, and remained within the stated reference range. The mean plasma total antioxidant status levels were 3-fold increased in the established diabetic group compared to control group values.

#### **4.2.5 Plasma $\alpha$ -tocopherol (vitamin E) concentration (Figure 4.2.5)**

Plasma vitamin E concentration ( $\mu\text{mol/l}$ ) were also significantly decreased in the established diabetic group (GP2) subjects compared with values from both recently diagnosed diabetic group (GP1) and non-diabetic (Cont GP) (Cont GP vs GP1 vs GP2,  $P < 0.001$ ) group subjects. In the recently diagnosed diabetic group and control group plasma vitamin E concentrations were almost similar and within the stated laboratory

reference range. However, mean concentrations of plasma vitamin E in the established diabetic group were 4-fold and recently diagnosed diabetic group were 3-fold decreased compared with the control group values.

#### **4.2.6 Association between plasma vitamin E concentration and HbA<sub>1c</sub> levels in established diabetic group (Figure 4.2.6)**

Decreased plasma vitamin E concentration ( $\mu\text{mol/l}$ ) was correlated with increased HbA<sub>1c</sub> (%) in the established diabetic group (GP2) as observed in the present study ( $n = 20$ ,  $r^2 = 0.3678$ ,  $y = 0.8292x - 18.441$ ,  $P < 0.001$ ).

#### **4.2.7 Association between plasma vitamin E concentration and urinary TBARS levels in established diabetic group (Figure 4.2.7)**

Plasma vitamin E concentrations ( $\mu\text{mol/l}$ ) were inversely and significantly correlated with increased urinary TBARS levels ( $\mu\text{mol/l}$ ) in the established diabetic group subjects ( $n = 20$ ,  $r^2 = 0.5024$ ,  $y = 0.3266x - 6.8581$ ,  $P < 0.0001$ ).

#### **4.2.8 Erythrocyte glutathione peroxidase (E-GSH-Px) levels (Figure 4.2.8)**

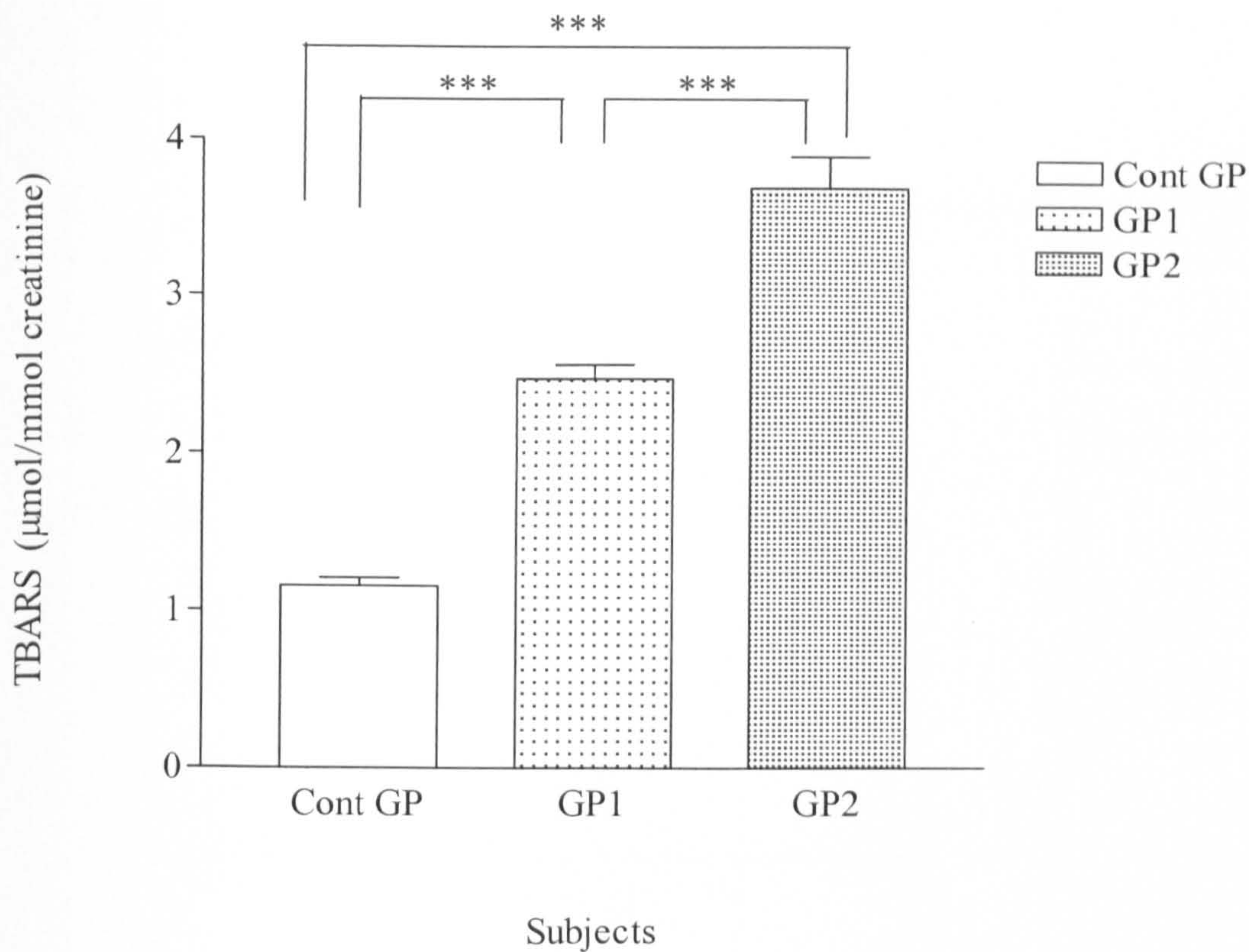
Decreased erythrocyte glutathione peroxidase levels (U/g Hb) were observed in both the established diabetic group (GP2) and recently diagnosed diabetic group (GP1) compared with control group (Cont GP) values (Cont GP vs GP1 vs GP2  $P < 0.001$ ). In the established diabetic group, erythrocyte glutathione peroxidase levels were significantly



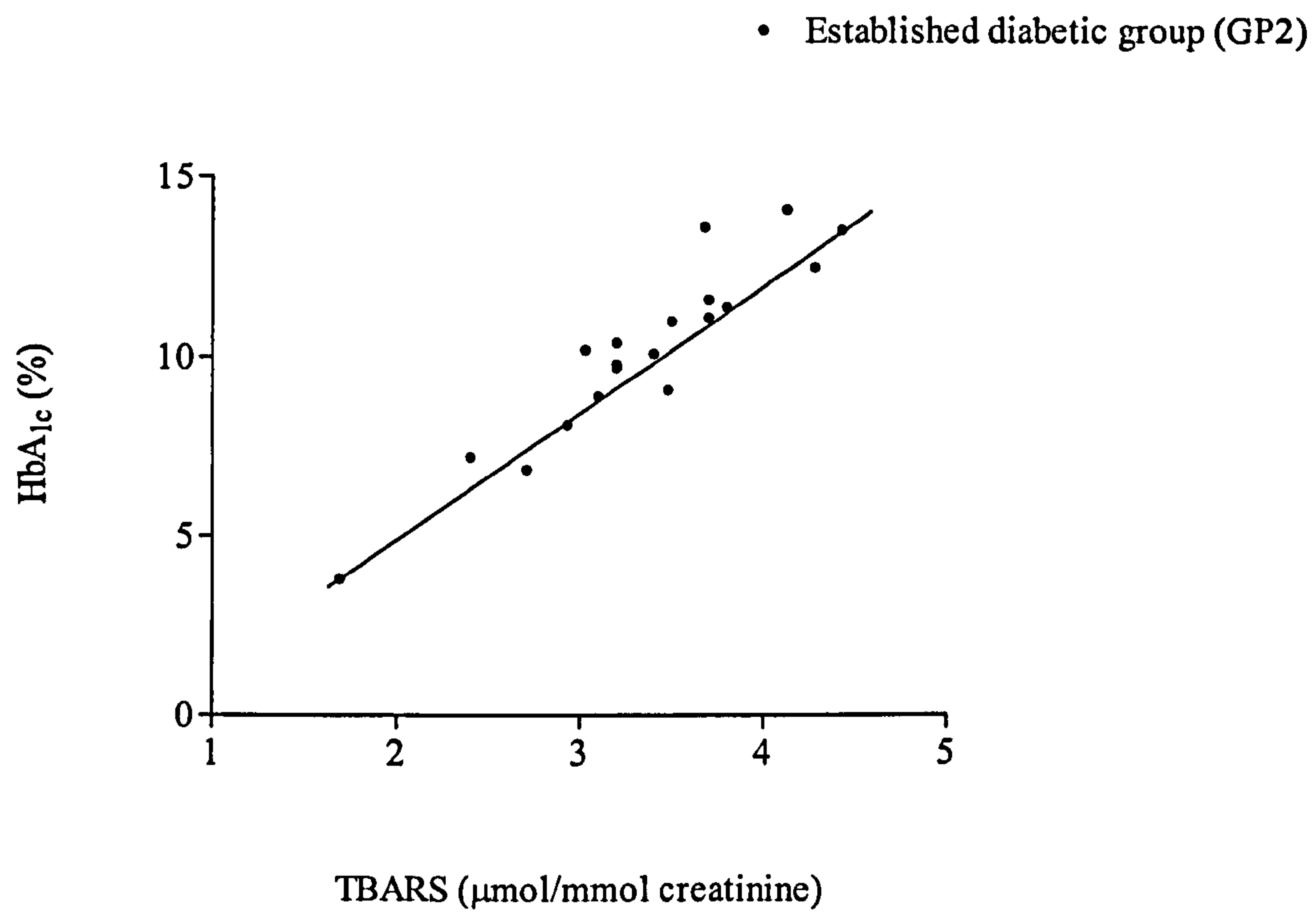
decreased when compared to both recently diagnosed diabetic and control groups ( $P < 0.001$ ). Both recently diagnosed diabetic group and control group erythrocyte glutathione peroxidase levels remained within the standard reference range. The mean erythrocyte glutathione peroxidase levels in the established diabetic group were 3-fold and 2.5-fold decreased compared with the control group and recently diagnosed diabetic group respectively.

#### **4.2.9 Plasma glutathione peroxidase (P-GSH-Px) activity (Figure 4.2.9)**

Plasma glutathione activity (U/l) was also decreased in both established diabetic group (GP2) and recently diagnosed diabetic group (GP1) compared to control group (Cont GP) values (Cont GP vs GP1 vs GP2  $P < 0.001$ ). Although recently diagnosed diabetic group plasma glutathione activities demonstrated a decreased trend, both recently diagnosed diabetic and control groups plasma glutathione values remained within the stated reference range. The mean plasma glutathione peroxidase activities in the established diabetic group were 3-fold and 2.5-fold decreased compared with the control group and recently diagnosed diabetic group, respectively.

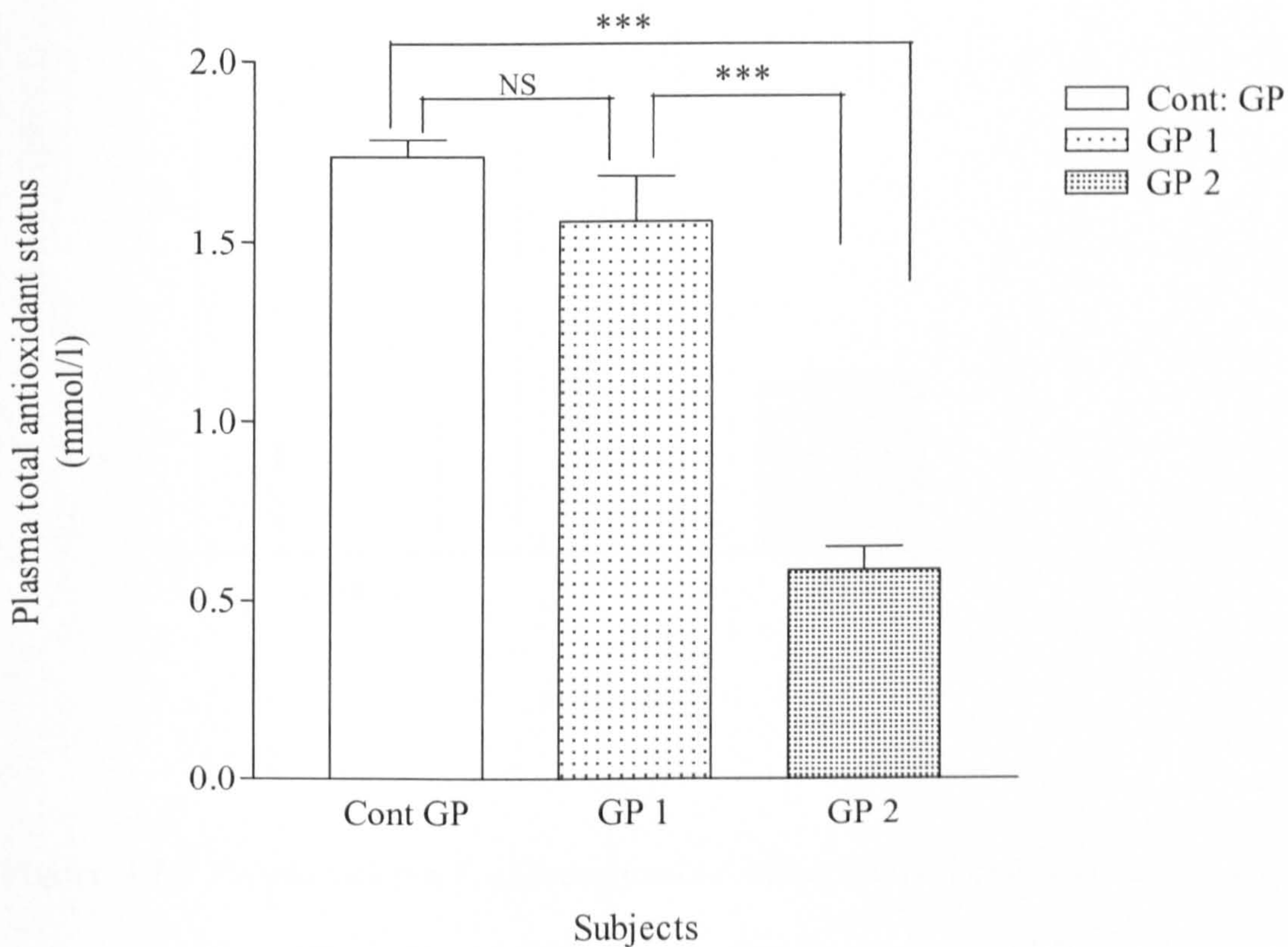


**Figure: 4.2.2** Urinary TBARS levels ( $\mu\text{mol}/\text{mmol creatinine}$ ) in control group (Cont GP), GP1 and GP2 subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ ).

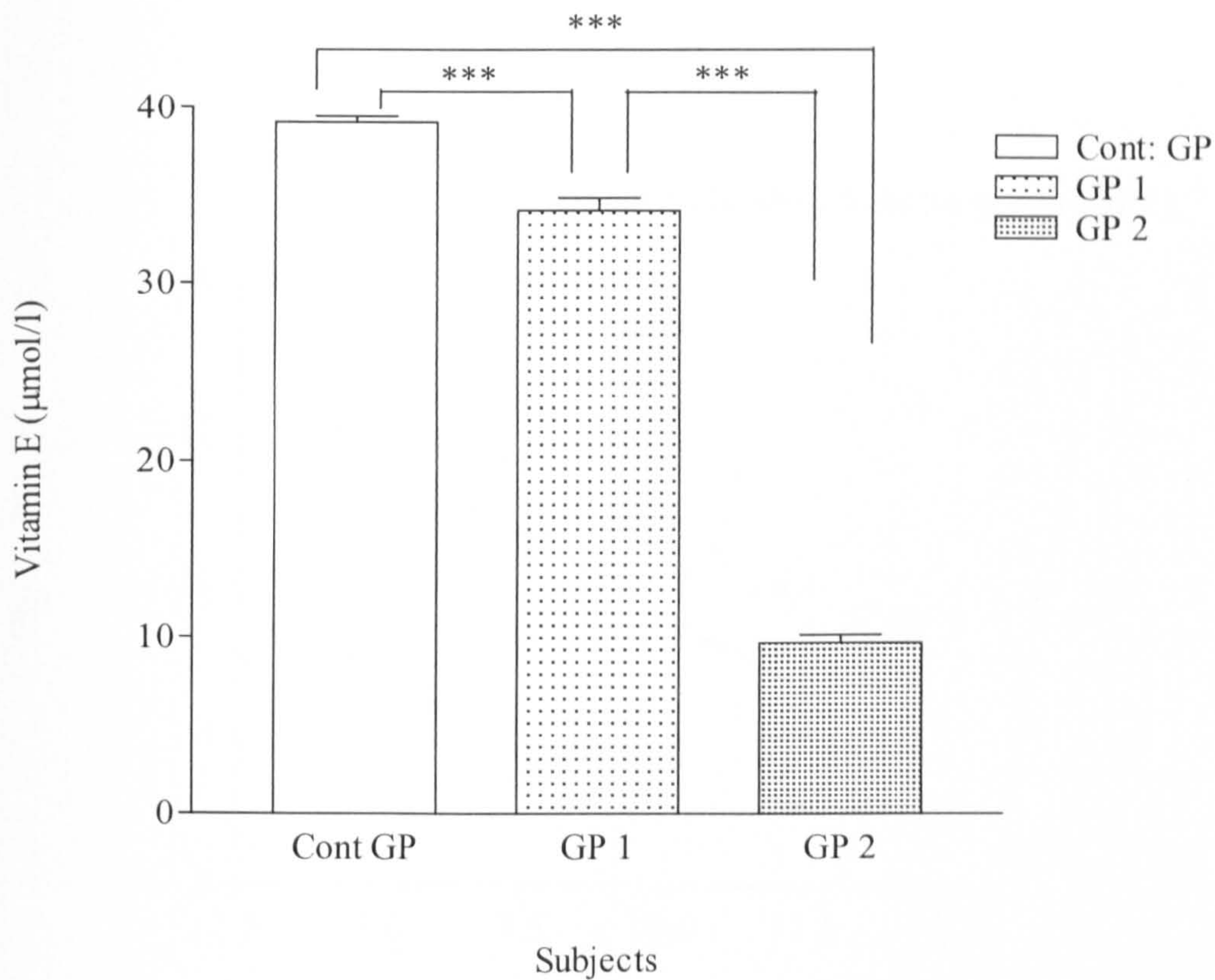


**Figure: 4.2.3** Correlation between urinary TBARS (μmol/mmol creatinine) and HbA<sub>1c</sub> (%) levels in the established diabetic group subjects (n = 20,  $r^2 = 0.894$ ,  $y = 3.68.2x - 2.1447$ ,  $P < 0.0001$ ).

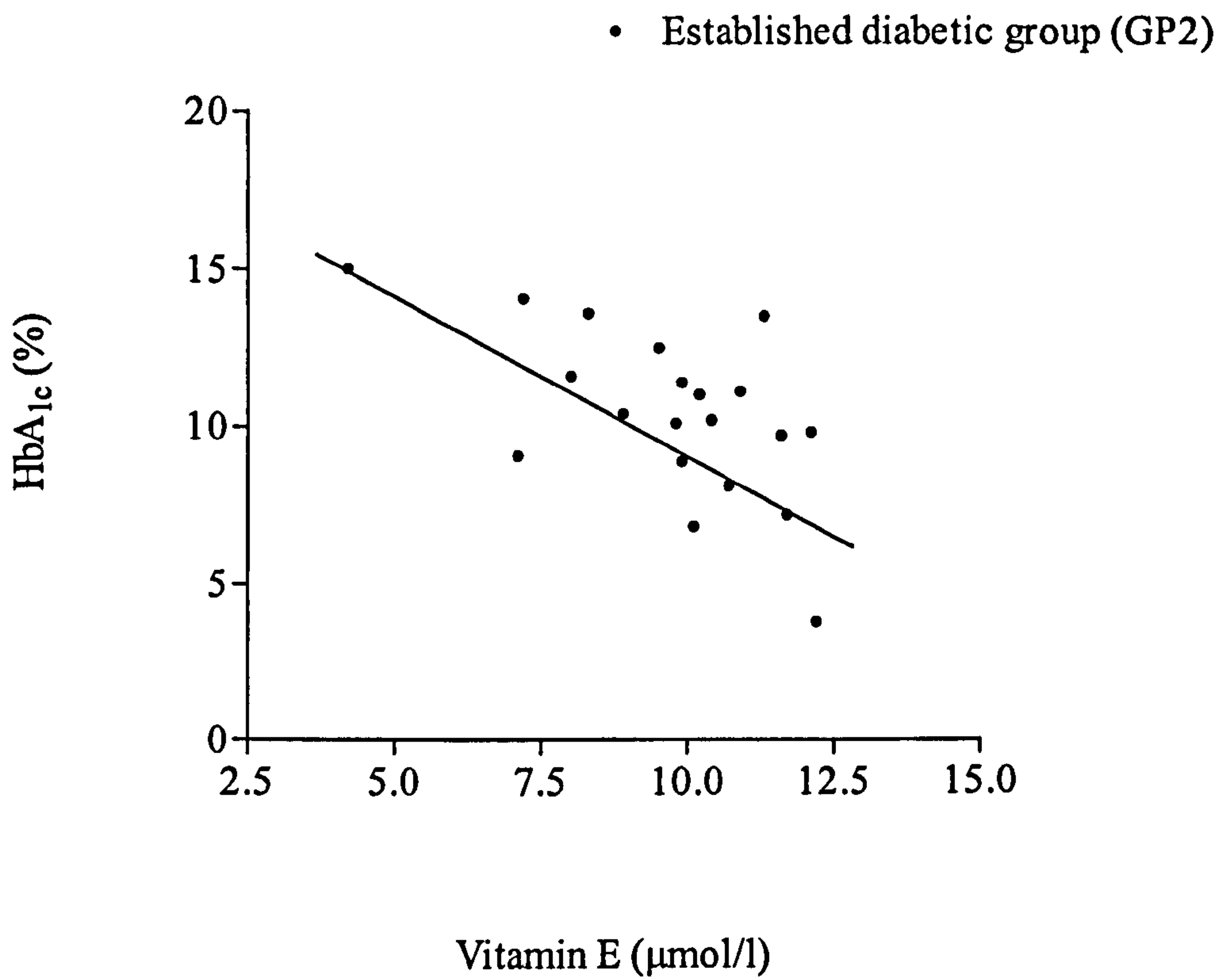




**Figure: 4.2.4** Plasma total antioxidant status (mmol/l) in control group (Cont GP), GP1, GP2 subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).

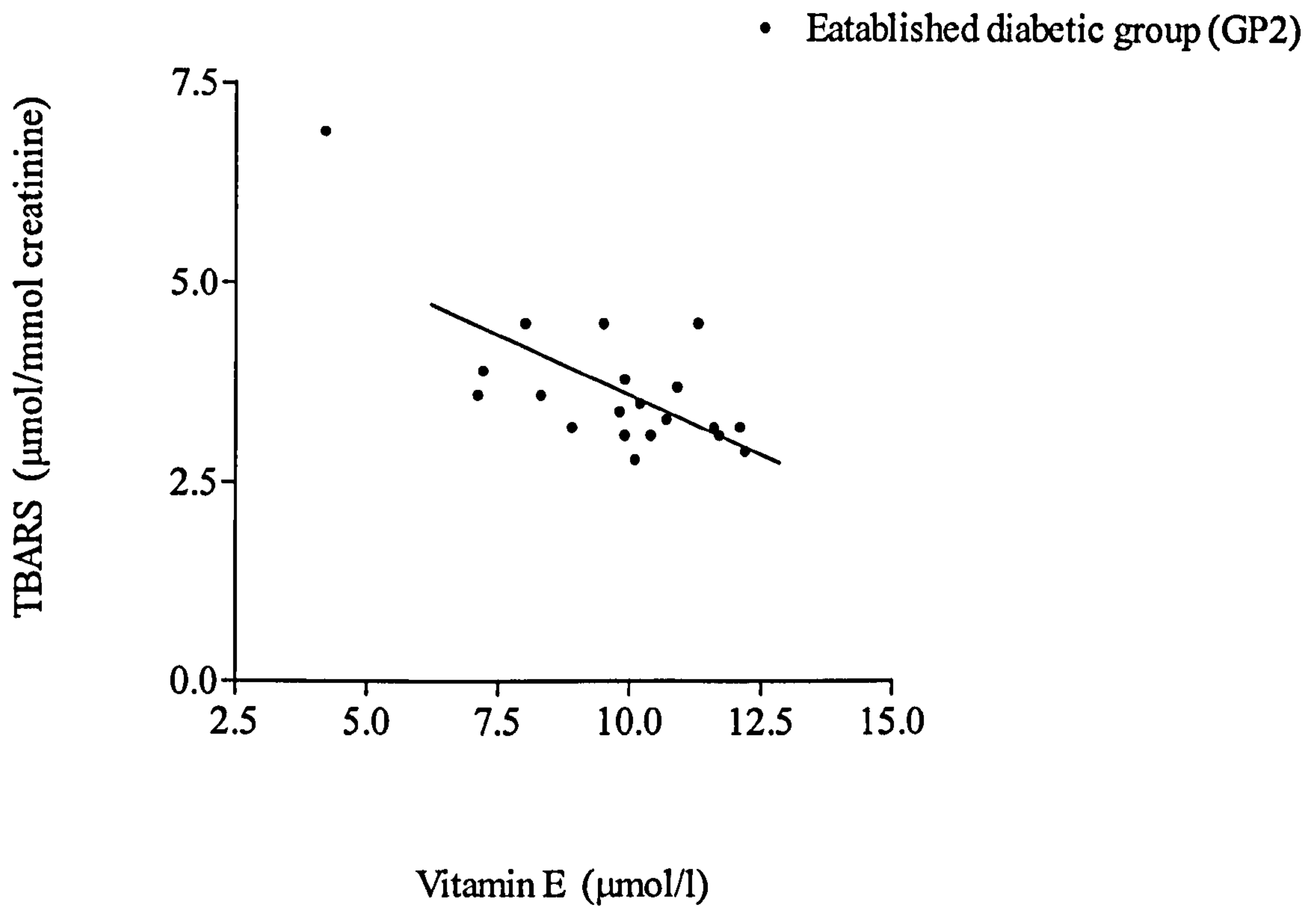


**Figure 4.2.5** Plasma vitamin E ( $\alpha$ -tocopherol) concentrations ( $\mu\text{mol/l}$ ) in control group (Cont GP), GP1 and GP2 subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ ).

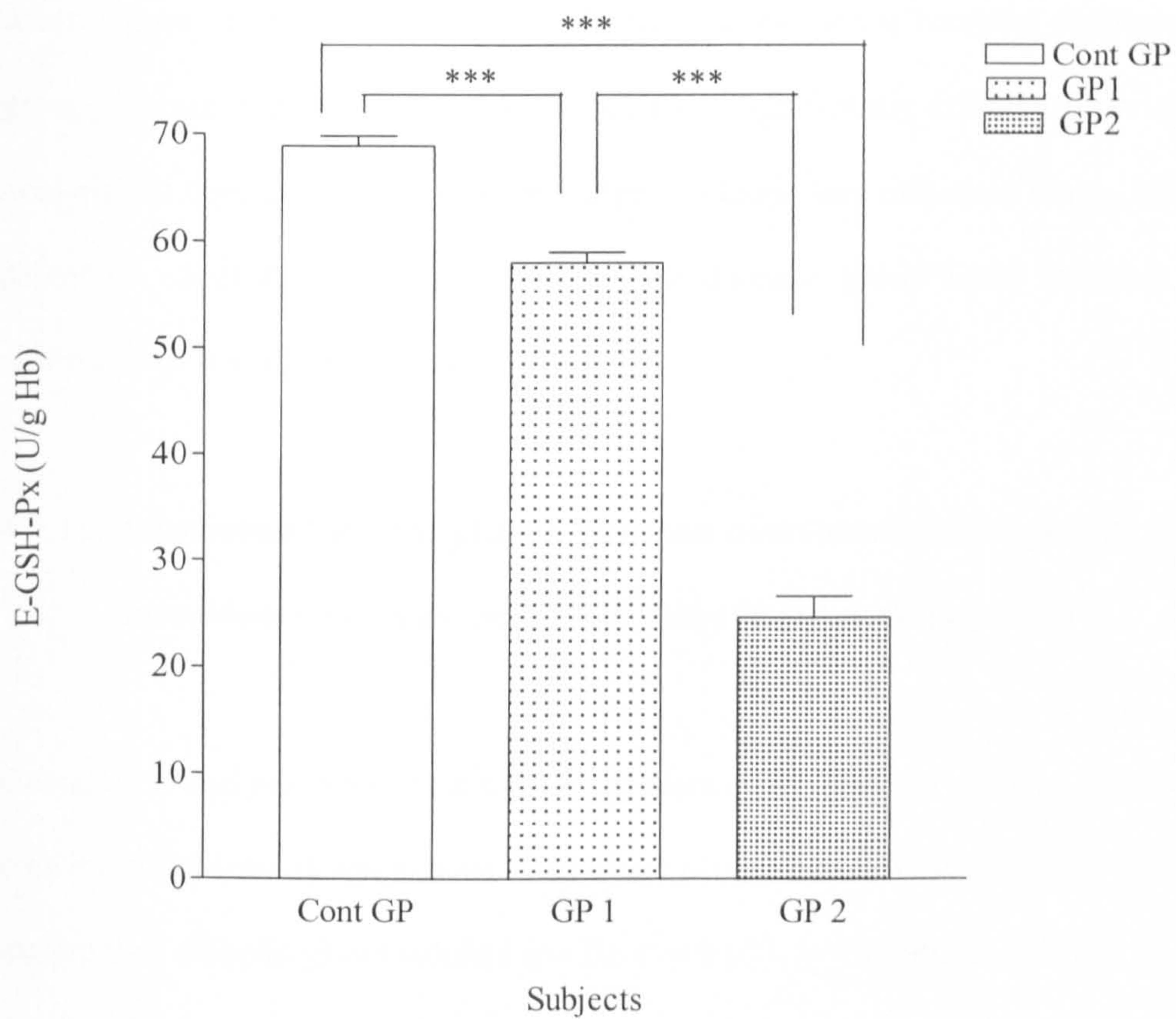


**Figure: 4.2.6** Correlation between plasma vitamin E concentration (µmol/l) and HbA1c (%) levels in the established diabetic group (GP2) subjects (n = 20,  $r^2 = 0.3678$ ,  $y = -0.8292x + 18.441$ ,  $P < 0.001$ ).





**Figure 4.2.7** Correlation between plasma vitamin E concentration ( $\mu\text{mol}/\text{l}$ ) and urinary TBARS ( $\mu\text{mol}/\text{mmol creatinine}$ ) levels in the established diabetic group (GP2) subjects.  $n = 20$ ,  $r^2 = 0.896$ ,  $y = -0.3266x - 6.8581$ ,  $P < 0.0001$ .



**Figure 4.2.8** Erythrocyte glutathione peroxidase (E-GSH-Px) levels (U/g Hb) in control (Cont GP), GP1 and GP2 groups. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ ).

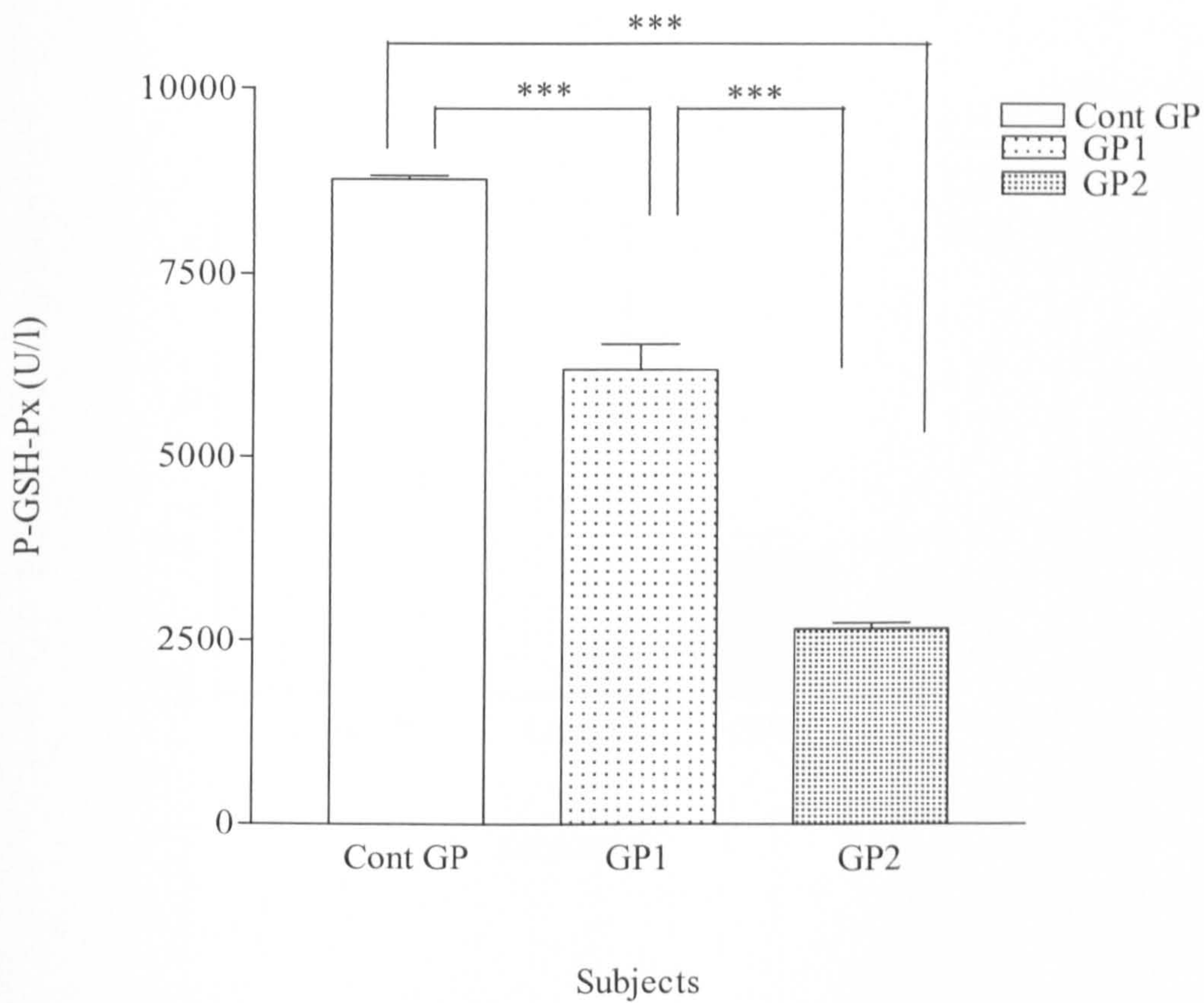
#### **4.2.10 Plasma selenium concentration (Figure 4.2.10)**

Plasma selenium concentrations ( $\mu\text{mol/l}$ ) were significantly decreased in the established diabetic group (GP2) compared to both GP1 (GP2 vs GP1,  $p < 0.001$ ) and non-diabetic control group (GP2 vs Cont GP,  $p < 0.001$ ) values. In the recently diagnosed diabetic group, plasma selenium concentrations were not significantly different from values in the non-diabetic control group, and were within the laboratory reference range. Mean plasma selenium concentrations in the established diabetic group were reduced by 4-fold compared to non-diabetic control values.

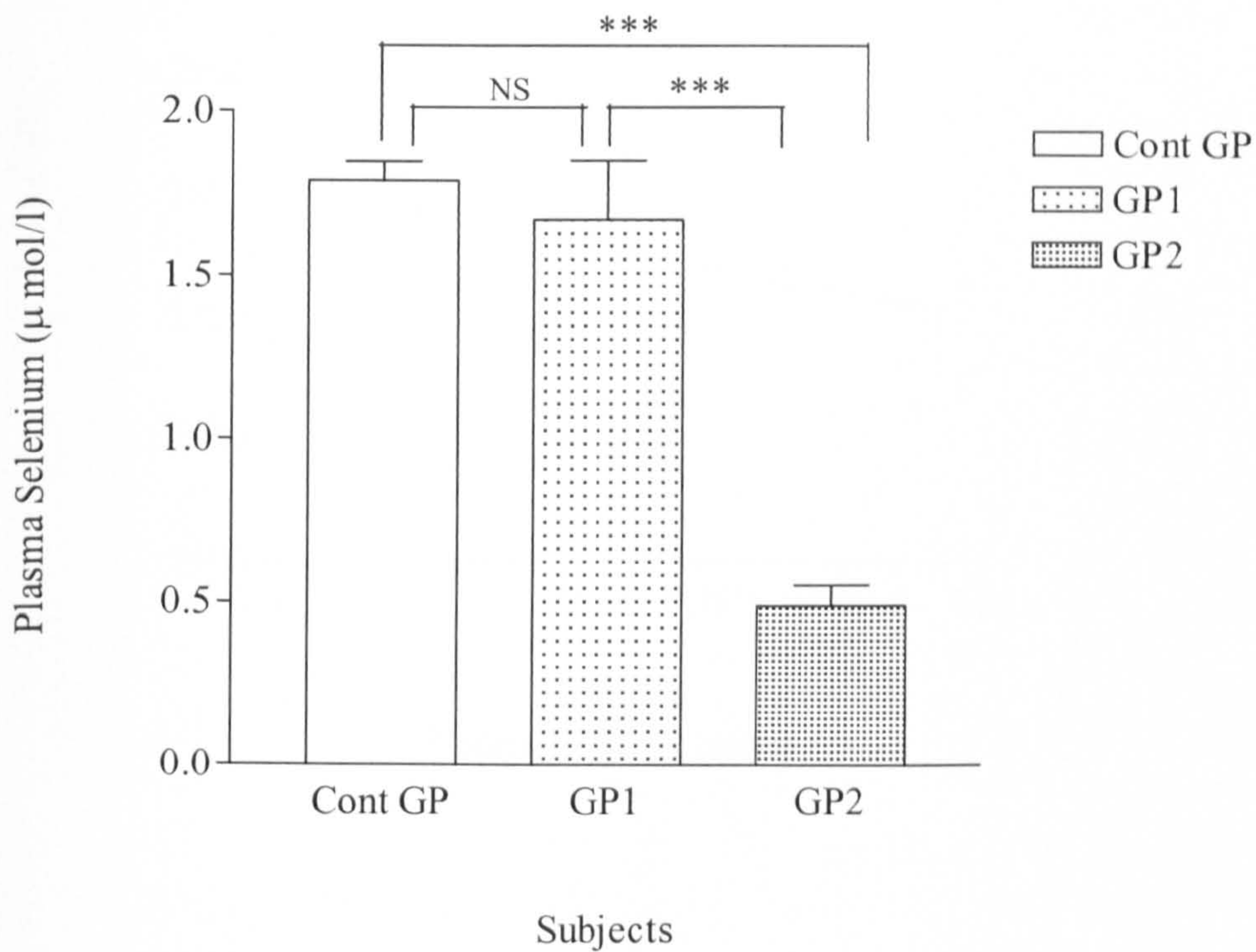
#### **4.2.11 Association between plasma selenium concentration and plasma glutathione peroxidase activity in established diabetic group (Figure 4.2.11)**

Correlation analysis revealed a significant association between reduced plasma selenium concentration ( $\text{mol/l}$ ) and plasma glutathione peroxidase (P-GSH-Px) activity ( $\text{U/l}$ ) in the established diabetic group values ( $n = 20$ ,  $r^2 = 0.633$ ,  $y = 98.908x - 2801.1$ ,  $P < 0.0001$ ).

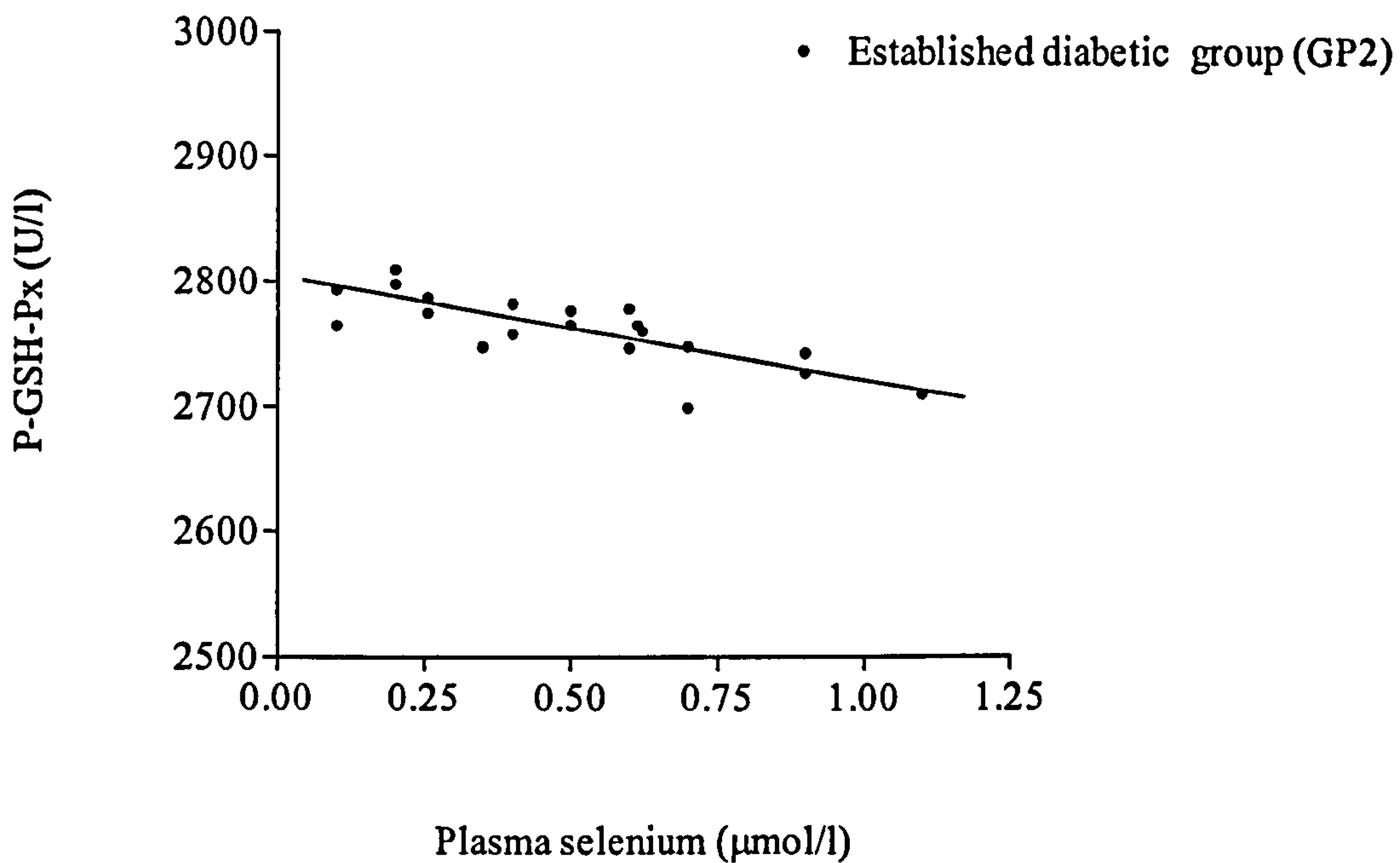




**Figure 4.2.9** Plasma glutathione peroxidase (P-GSH-Px) activity (U/l) in control group (Cont GP), GP1 and GP2 subjects. Results (mean ± SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ ).

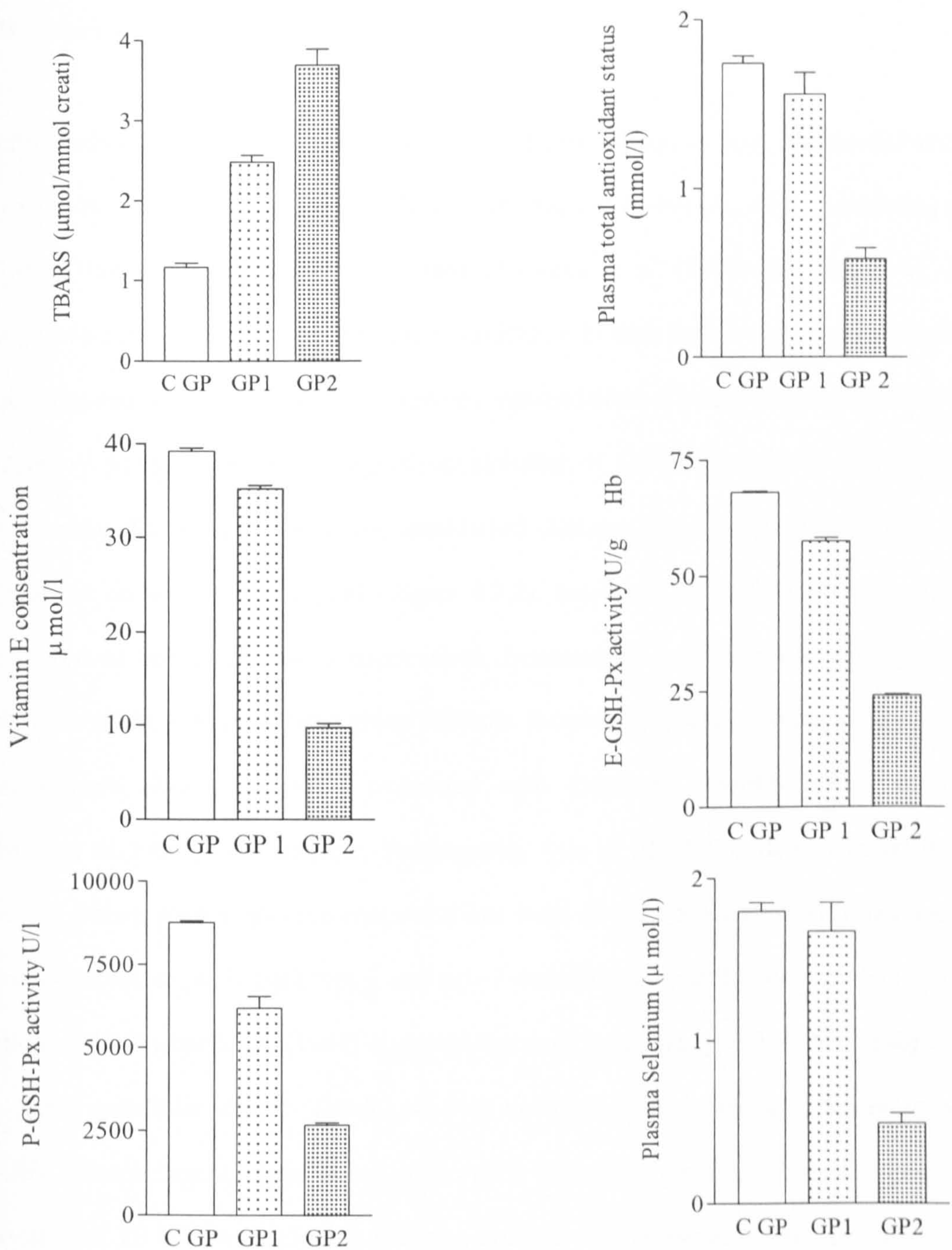


**Figure 4.2.10** Plasma selenium concentrations ( $\mu\text{mol/l}$ ) in control group (Cont GP), GP1 and GP2 subjects. Results (mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).



**Figure 4.2.11** Correlation between plasma selenium concentration (μmol/l) and plasma glutathione peroxidase (P-GSH-Px) activity (U/l) in the established diabetic group (GP2) subjects (n = 20,  $r^2 = 0.633$ ,  $y = -78.908x - 2801.1$ ,  $P < 0.0001$ ).





**Figure 4.2.12** Summary of the lipid peroxidation and antioxidant biochemical markers.  
 C GP: control group, GP1: recently diagnosed diabetic group,  
 GP2: established diabetic group.

### 4.3 Discussion

Several studies have demonstrated enhanced lipid peroxidation in both human diabetics and experimental diabetic animal models. (Armstrong and Al-Awadi, 1991; Armstrong *et al*, 1992; Hartnett *et al*, 2000). The studies of Akkus *et al*, (1996) and Santini *et al*, (1997) have demonstrated increased lipid peroxidation in both type 1 and type 2 diabetes when compared with non-diabetic age and sex matched control subjects using a TBARS-MDA assay. In the present study, significant elevation of TBARS levels was observed in both recently diagnosed diabetic and established diabetic group subjects compared to non-diabetic control group subjects (Figure 4.2.2). Increased TBARS levels associated with increased fasting glucose concentrations, fructosamine and glycated haemoglobin levels were noted in both diabetic group subjects. Increased glycated haemoglobin levels (HbA<sub>1c</sub>) were also significantly correlated with increased TBARS levels in the established diabetic group subjects. Furthermore, Sato *et al*, (1979) demonstrated that increased fasting plasma glucose concentrations were directly associated with increased TBARS concentrations in both type 1 and type 2 diabetics compared to control values. In addition, Griesmacher *et al*, (1994) observed that both type 1 and type 2 diabetic subjects with good metabolic control (HbA<sub>1c</sub> <6.5%) demonstrated significantly lower urine TBARS concentrations than those with poor metabolic control, but all groups demonstrated TBARS values higher than non-diabetic control subjects. The results of the present studies are consistent with the findings of the above and other studies that enhanced lipid peroxidation is present in subjects with diabetes as measured by the TBARS assay (Rabini *et al*, 1994; Akkus *et al*, 1996). Increased glycated protein and increased TBARS levels in the established diabetic group suggesting that poor metabolic



control and persistent hyperglycaemia is associated with increased lipid peroxidation (Rabini *et al*, 1994; Griesmacher *et al*, 1994; Jain, 2000). Furthermore, the accumulation of glycosylation products (i.e., HbA<sub>1c</sub>) may have a predictive value for the presence of an enhanced rate of lipid peroxidation in subjects with poor glycaemic control. (Jain, 2000). Consequently, the above observations are consistent with the suggestion that glycation products can be associated with the generation of free radicals, and subsequently enhanced lipid peroxidation (Halliwell and Gutteridge, 1998).

Clinical and experimental diabetic studies have also demonstrated a relationship between increased lipid peroxidation and hyperlipidaemia (Nishigaki *et al*, 1981; Griesmacher *et al*, 1994; Mooradian, 1991; Esterbauer *et al*, 1992; Nacitarhan *et al*, 1995;). The present study also revealed a close association between increased TBARS excretion and dislipidaemia, as shown by elevation of cholesterol, triglycerides and LDL concentrations in the established diabetic group. However, this association was not statistically significant in the present study. Furthermore, controversial results have been reported regarding the association between cholesterol, triglycerides and LDL with circulating TBARS levels. Nacitarhan *et al*, (1995) demonstrated higher concentrations of TBARS in hyperlipidaemic diabetics (cholesterol > 6mmol/l and triglycerides concentration > 2mmol/l) compared to nonlipidaemic diabetics and a significant correlation between TBARS and lipid concentration was also noted. Conversely, Griesmacher *et al*, (1994) did not observe any such correlation between TBARS and lipid concentrations. Consequently, these observations suggest that the concentration of TBARS does not depend only the availability of substrate for lipid peroxidation (Mooradian, 1991). This



would suggest that an increase in the total amount of circulating lipids plays an additional role in the enhanced lipid peroxidation observed in diabetes (Griesmacher *et al*, 1994). In subjects with moderately severe diabetes, LDL catabolism is impaired resulting in an increased proportion of triglycerides. Furthermore, the increase in the concentration of small, dense LDL in serum, their glycation and oxidative modification (Brownlee *et al*, 1984) contribute to the increase in total serum LDL observed in poorly controlled diabetic subjects. *In vitro* studies have also demonstrated that the incubation of lipids with glycated proteins resulted in an elevation of lipid peroxidation (Lyons, 1992). Moreover, it has been suggested that lipid-advanced glycosylation and oxidation increased during the persistent hyperglycaemia associated with diabetes, supports the concept that AGE oxidation plays an important role in initiating lipid peroxidation *in vivo* (Bucala *et al*, 1993).

An inverse association was observed between total antioxidant status and plasma glucose concentration, HbA<sub>1c</sub>, in the established diabetic group subjects. Similarly, the studies of Johnston and Miller, (1998) and Vantuyghem *et al*, (2000) have also noted that lipid peroxidation was increased and total antioxidant status significantly decreased in poorly controlled diabetes. The reduction in total antioxidant status in diabetics has been significantly associated with increasing HbA<sub>1c</sub> levels and also with the duration of diabetes (Valubliji *et al*, 2001). These observations are further supported by the evidence that increased HbA<sub>1c</sub> can influence the generation of superoxide radicals, that may ultimately lead to increased lipid peroxidation (Griesmacher *et al*, 1994; Eriksson *et al*, 1996; Jain *et al*, 1999). Although significantly increased TBARS and decreased total

antioxidant capacity were observed in the established diabetic group in the present study, a significant correlation between these two parameters was not observed. However, a positive correlation between total antioxidant status and TBARS in other studies (Asayama *et al*, 1993; Sundaram *et al*, 1996) suggests that extracellular antioxidants were unable to provide an effective protection against lipid peroxidation in subjects with inadequate glycaemic control. Consequently, the results presented here are consistent with other similar studies that suggest persistent hyperglycaemia, poor metabolic control and increased non-enzymatic glycation can be associated with increased lipid peroxidation via enhanced free radical production, resulting from impaired antioxidants status (Sundaram *et al*, 1996; Maxwell, 1997; Sirinivasan *et al*, 1997) in established diabetic subjects.

Vitamin E concentrations were significantly decreased only in the established diabetic group compared to both recently diagnosed diabetic and control group values (Figure 4.2.5). Furthermore, a significant apparent correlation between vitamin E concentration, HbA<sub>1c</sub> and TBARS levels were also observed in the established diabetic group subjects (Figure 4.2.6, 4.2.7). In addition, in a study by Girelli *et al*, (1992) an inverse association between decreased vitamin E and increased total cholesterol, triglycerides and LDL concentrations was noted in the established diabetic group. However, no correlation was observed between TBARS and LDL concentrations in this group. Conversely, Reavan *et al*, (1995) demonstrated a significant correlation between vitamin E and LDL concentrations in subjects with diabetes. Decreased vitamin E was associated with a decreased susceptibility of LDL to oxidation in comparison with placebo

supplementation of 600 IU vitamin E/day for 4 weeks to type 2 diabetic which reduced the observed elevated levels of TBARS. There is also sufficient evidence for vitamin E deficiency in the diet of diabetic subjects which subsequently results in decreased plasma vitamin E concentrations compared with values observed in non-diabetic controls (Salonen *et al*, 1995). Consequently, consistence with these observations, the results of present study suggest that persistent hyperglycaemia, increased glycated protein and poor metabolic control can precipitate influenced increase lipid peroxidation in diabetic subjects with inadequate vitamin E levels that may ultimately lead to enhanced oxidative stress (Jain, 1989; Leonhardt, 1996; Santini, 1997).

Several studies, including both human (Aaseth and Stoa-Birketvedt, 2000; Varvarovska *et al*, 2003) and experimental (Mukherjee *et al*, 1998), have demonstrated a significant depletion of glutathione peroxidase in the diabetic state. In the present study, both erythrocyte and plasma glutathione peroxidase levels were significantly decreased in the established diabetic group compared to both recently diagnosed diabetic and non-diabetic control groups values, (Figure 4.2.8). However, no significant correlation was observed between erythrocyte and plasma glutathione peroxidase levels in studies by Bennefont-Rousselot *et al*, (1997). In present studies the established diabetic group demonstrated, increased fasting glucose concentration, HbA<sub>1c</sub>, total cholesterol, tryglycerides, LDL, TBARS, decreased total antioxidant status and vitamin E levels associated with a decrease in both erythrocytes and plasma glutathione peroxidase levels. However, correlation analysis did not reveal any significant associations between these parameters and glutathione peroxidase levels. However, Jain and McVie, (1994) and Jain *et al*,



(2000) both demonstrated a significant correlation between increased HbA<sub>1c</sub>, MDA and decreased glutathione peroxidase levels suggesting an important association between poor metabolic control and oxidative stress in subjects with diabetes. Conversely, several other studies have reported increased cellular glutathione peroxidase activity in subjects with diabetes and its complications, in both human and experimental animal models (Kakkar *et al*, 1997; Ceriello *et al*, 2000). This evidence may have important consequences with regard to glucose-induced oxidative insult to the cell. Hydrogen peroxide, produced during the lipid peroxidation process, is converted to water in peroxisomes by the antioxidant enzyme catalase (CAT) and in the cytoplasm by glutathione peroxidase. These antioxidant enzymes protect the cell from oxidative stress but the threshold of protection dramatically depends on the total antioxidant capability. Consequently, increased glutathione peroxidase activity may suggest a compensatory response by the defence mechanisms in response to increased oxidative stress (Ceriello *et al*, 2000). However, the results of the present investigation are consistent with previous studies on depletion of cellular glutathione levels in diabetic states as reflected by reduced glutathione peroxidase activity in poorly controlled diabetes (Aaseth and Stoa-Birketvedt, 2000). Furthermore, vitamin E supplementation has been demonstrated to increase cellular glutathione concentration, decrease HbA<sub>1c</sub>, MDA concentrations and may reduced the incidence of vascular disease in diabetics (Jain *et al* 2000). Several mechanisms contribute to glutathione deprivation in subjects with diabetes and its complications and the abundance of glucose involving activation of the polyol pathway has been suggested as enhanced oxidative stress is observed in the diabetic state (Rosen *et al*, 2001).

The antioxidant ability of selenium resides in the active side chain of the seleno-enzyme glutathione peroxidase and the importance of selenium as an essential trace element is probably due to its presence in this enzyme. Significantly decreased plasma selenium concentrations were noted in the established diabetic group subjects. The results from recently diagnosed diabetic and control groups were within reference intervals. As previously described, increased fasting glucose concentration, HbA<sub>1c</sub>, total cholesterol, triglycerides, LDL, TBARS and decreased total antioxidant status, vitamin E and erythrocyte glutathione peroxidase levels were all associated with decreased selenium concentration in the established diabetic group. However, correlation analysis did not reveal any significant associations between these parameters and selenium concentrations. The above observation is consistent with other studies suggesting that the deficiency of selenium would tend to increase oxidative stress in subjects with persistent hyperglycaemia (Schlienger *et al*, 1988; Meydani *et al*, 1988; Bennefont-Rousselot *et al*, (1997). Furthermore, a correlation between both low selenium concentration and plasma glutathione peroxidase activity (Bennefont-Rousselot *et al*, 1997) was observed in the established diabetic group subjects. Plasma glutathione peroxidase originates from both the cells of the proximal tubular epithelium, and the parietal cells of Bowman's capsule in the kidney (Mony and Larras-Regard, 2000). Consequently, the kidney play a major role in selenium and plasma glutathione peroxidase metabolism (Mony and Larras-Regard, 2000). The effects of selenium on diabetic complications, an association between low plasma selenium concentration and a high index of micro and macroangiopathy has previously been observed in subjects with diabetes. Furthermore, dietary supplementation improved glycaemic control normalised the renal lipid peroxide levels of the kidney and

normalised hyperfiltration (Douillet *et al*, 1999). This reduced oxidative stress would also be involved in the reduction of renal lesions since oxidative stress has been implicated in the development of diabetic nephropathy (Douillet *et al*, 1999).

In contrast, in the recently diagnosed diabetic group, the presence of increased TBARS and stable antioxidants such as total antioxidant status, vitamin E, both erythrocytes and plasma glutathione peroxidase and selenium levels suggests that the presence of recent onset hyperglycaemia may contribute to increased lipid peroxidation via free radical production as an early event in the pathogenesis of diabetic nephropathy. However, while at this stage the antioxidant defence mechanisms are still sufficient to counteract their activity and balance (Asayama *et al*, 1993), in the established diabetic group subjects, defence mechanisms are overwhelmed because of the increased severity of oxidative stress. Furthermore, consistent with this observation made in the present study and supported by other studies persistent hyperglycaemia is the major potential determinant of increased free radical production by stimulating several biochemical pathways, producing enhanced oxidative damage in subjects with diabetes mellitus. Consequently, the association between oxidative stress, progression and the development of diabetic kidney dysfunction has been evaluated in order to further establish the free radical mediated oxidative stress plays in pathogenesis of diabetic nephropathy.



## **CHAPTER FIVE**

### **AN INVESTIGATION OF GLOMERULAR AND TUBULAR FUNCTION IN A DIABETIC POPULATION**

## 5. Introduction

It is well established that the free radical mediated oxidative stress associated with persistent hyperglycaemia plays an important role in the development of diabetic nephropathy. Furthermore, it has been demonstrated that oxidative stress is increased in the diabetic kidney, presumably before onset of any clinical signs of nephropathy. High glucose concentrations have been shown to increase oxidative stress in target cells *in vitro*, and oxidative stress has a relevant effect on target cells in both *in vivo* and *in vitro* (Fukui *et al*, 1992; Ha and Kim, 1999). The early changes in diabetic nephropathy are characterised by increased kidney size, glomerular volume and kidney function and later on by the development of mesangial proliferation, accumulation of extracellular matrix and increased urinary albumin excretion (UAE). (Flyvbjerg *et al*, 2000). However, in the initial stages of diabetic nephropathy, clinical signs and symptoms may be vague or even absent for sometime.

In its early appearance, the first morphological alterations observed in the kidney are hypertrophy of both the glomerular and tubular elements; subsequently, a thinning of glomerular and tubular basement membranes is evident, with enhanced glomerular permeability to albumin also being observed (Christiansen *et al*, 1981). This leads to a progressive accumulation of extracellular matrix components in both the glomerular mesangium and in tubulointerstitial structures (Parving *et al*, 2000). Clinical and experimental studies have suggested that oxidative injury may alter the structure and biochemical functions of the diabetic kidney due to the effect of free radicals on both

glomerular and tubular structures and their function. However, the early recognition of renal insult remains controversial. Serum creatinine, the standard marker of glomerular function, is at best insensitive and represents a relatively late manifestation of diabetic nephropathy. The aims of this study was refer to investigate the use of other proteins, namely specific proteins, enzymes and serum components including cyctatin C and sialic acid, in the early recognition of kidney insult in a diabetic population. A second subsidiary aim was to relate any changes in these parameters indicating early renal insult to the antioxidant status present in the diabetic state as discussed in the previous chapter.

## **5.1 Materials and methods**

The study groups (Section 2.1.1), materials and methods (Section 2.4.1 to 2.4.9) are as described in Chapter 2.

## **5.2 Results**

### **5.2.1 Serum creatinine concentration**

Serum creatinine concentrations were similar in the recently diagnosed diabetic group, established diabetic group and control group subjects at  $96.0 \pm 6.0$ ,  $97.1 \pm 6.7$  and  $98.6 \pm 5.8$   $\mu\text{mol/l}$  (Mean $\pm$ SEM) respectively. All patients results were within the laborotary reference intervals (Men:< 115  $\mu\text{mol/l}$ , Women :<97  $\mu\text{mol/l}$ ).



### **5.2.2 Albumin:creatinine ratio (microalbuminuria) (Figure 5.2.2)**

In the established diabetic group (GP2) the albumin:creatinine concentration (mg/mmol creatinine) was significantly increased compared to values observed from both the recently diagnosed diabetic group (GP2 vs GP1,  $P < 0.001$ ) and control group (GP2 vs Cont GP,  $P < 0.001$ ) respectively. In the recently diagnosed diabetic group (GP1), the albumin:creatinine concentration (mg/mmol creatinine) was similar to that observed in the control group and both groups remained within the laboratory reference intervals ( $< 3.5$  mg/mmol creatinine). The mean albumin:creatinine concentration (mg/mmol creatinine) was 3-fold increased in the established diabetic group compared with both the recently diagnosed diabetic and control groups.

### **5.2.3 Association between albumin:creatinine ratio and glycated haemoglobin (HbA<sub>1c</sub>) (Figure 5.2.3)**

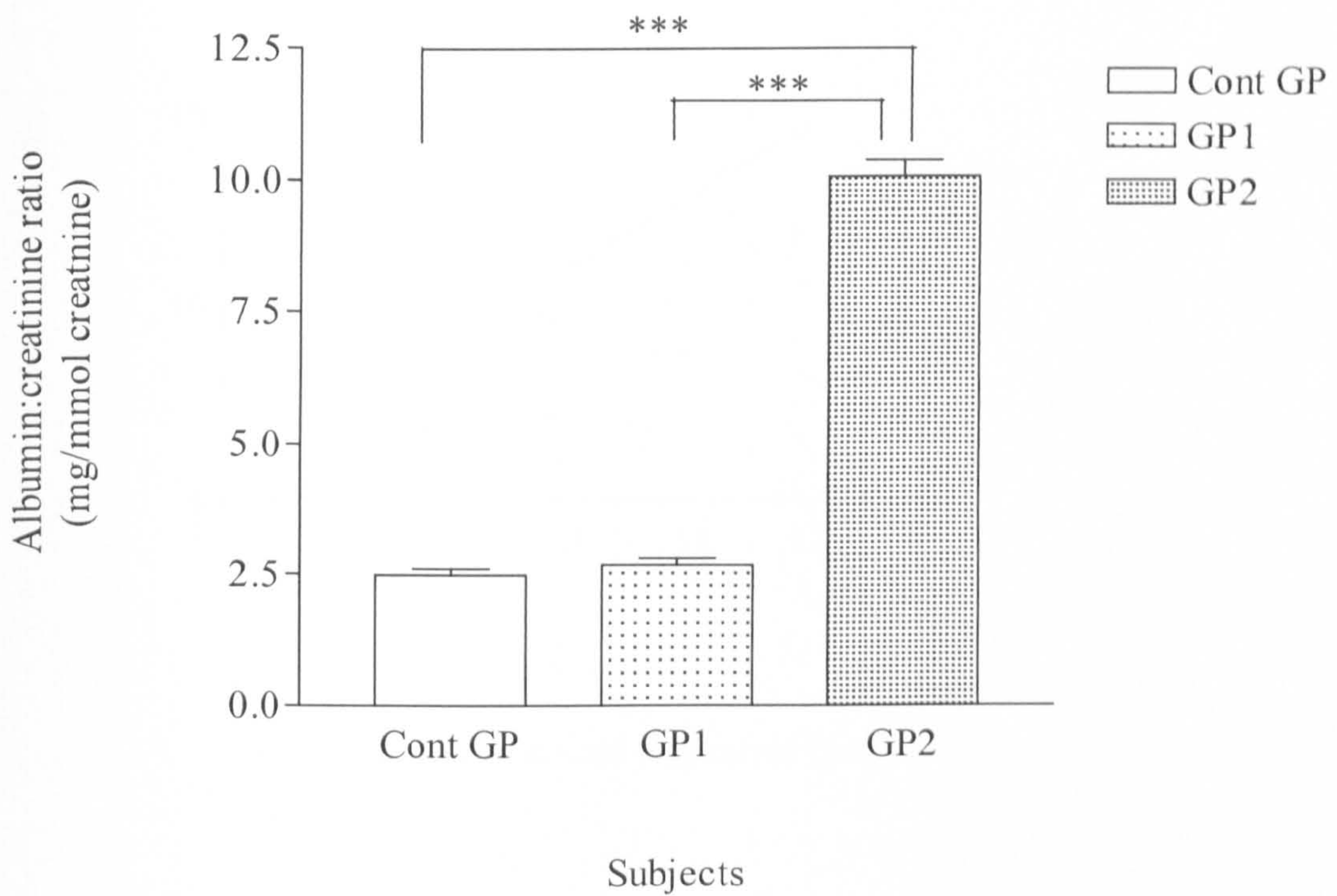
A significant correlation between albumin:creatinine ratio and glycated haemoglobin (HbA<sub>1c</sub>) values was observed in the established diabetic group (GP2).  $r^2 = 0.42$ ,  $y = 1.3313x - 3.0152$ ,  $P < 0.001$ .

#### **5.2.4 Serum sialic acid concentration (Figure 5.2.4)**

Serum sialic acid concentration (mmol/l) was significantly increased in the established diabetic group (GP2) compared to results observed from both the recently diagnosed diabetic group (GP1) and control group (GP2 vs GP1,  $P < 0.001$ , GP2 vs Cont GP,  $P < 0.001$ ) subjects. Serum sialic acid concentrations were similar in the control and recently diagnosed diabetic group and were within the laboratory reference interval (1.5-2.2 mmol/l). Furthermore, mean concentrations of sialic acid (mmol/l) in the established diabetic group were increased 1.5-fold compared with both results from the recently diagnosed diabetic and control groups.

#### **5.2.5 Serum cystatin C concentration (Figure 5.2.5)**

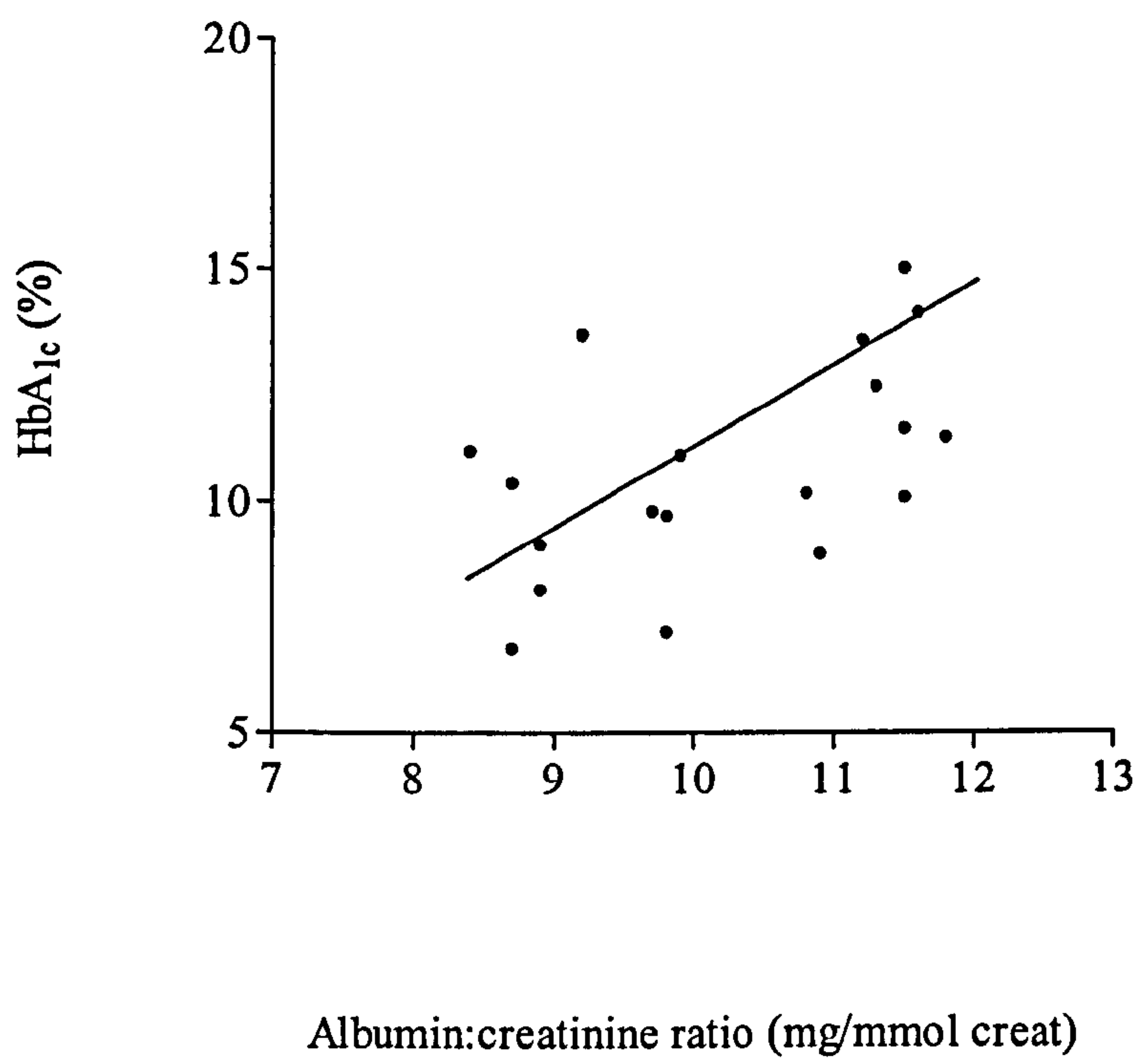
In the established diabetic group (GP2), significantly increased cystatin C concentration (mg/l) were observed compared to results from both the acute hyperglycaemic (GP2 vs GP1,  $P < 0.001$ ) and control groups (GP2 vs Cont GP,  $P < 0.001$ ). Although the acute hyperglycaemic group (GP1) demonstrated a decreased trend in cystatin C concentration compared to the control group (Cont GP) values, results were similar and within the stated reference range (0.7-1.2 mg/l). Mean serum cystatin C concentration (mg/l) in the established diabetic group were increased 1.5-fold compared to those observed in the recently diagnosed diabetic group.



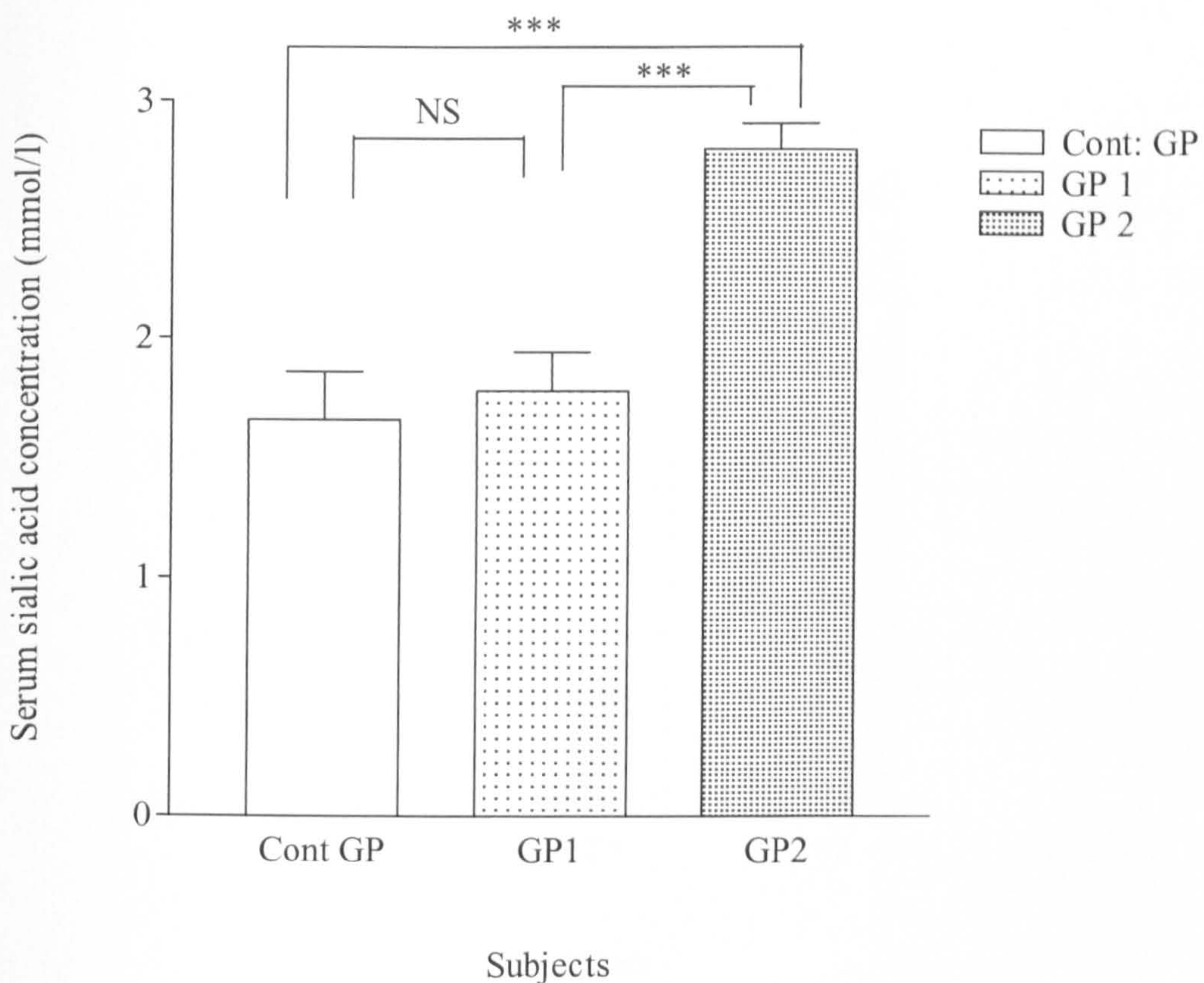
**Figure 5.2.2** Albumin:creatinine ratio (mg/mmol creatinine) in control group (Cont GP), GP1 and GP2 subjects. Results (Mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ ).



• Established diabetic group (GP2)

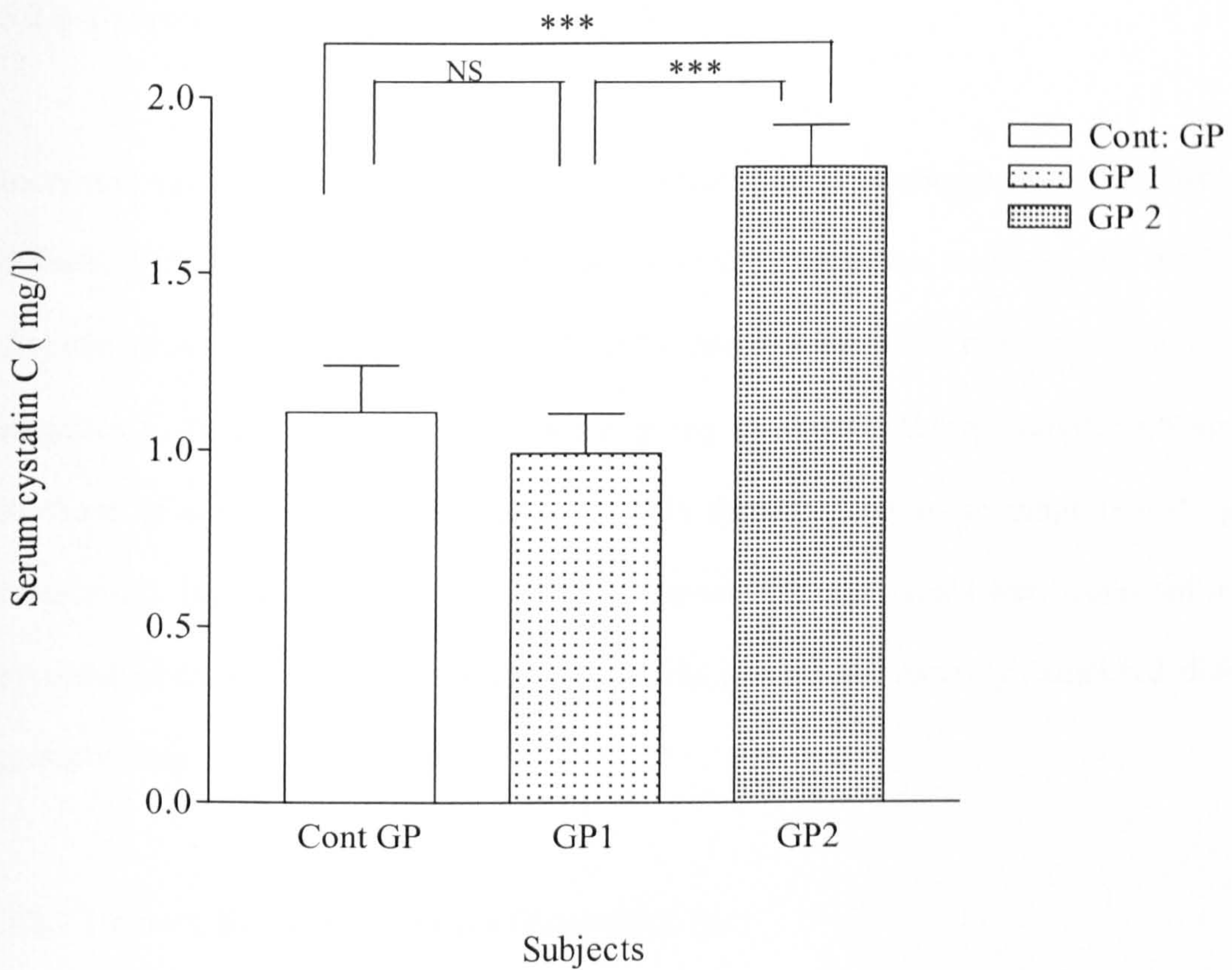


**Figure 5.2.3** Correlation between microalbuminuria (albumin:creatinine mg/mmol creatinine) and glycated haemoglobin (HbA<sub>1c</sub> %) in the chronic hyperglycaemic group (GP2) subjects (n = 20,  $r^2 = 0.42$ ,  $y = 1.3313x - 3.0152$ ,  $P < 0.001$ ).



**Figure 5.2.4** Serum sialic acid concentrations (mmol/l) in control (Cont GP), GP1 and GP2 subjects. Results (Mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).





**Figure 5.2.5** Serum cystatin C concentrations (mg/l) in control (Cont GP), GP1 and GP2 subjects. Results (Mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferrionie's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).

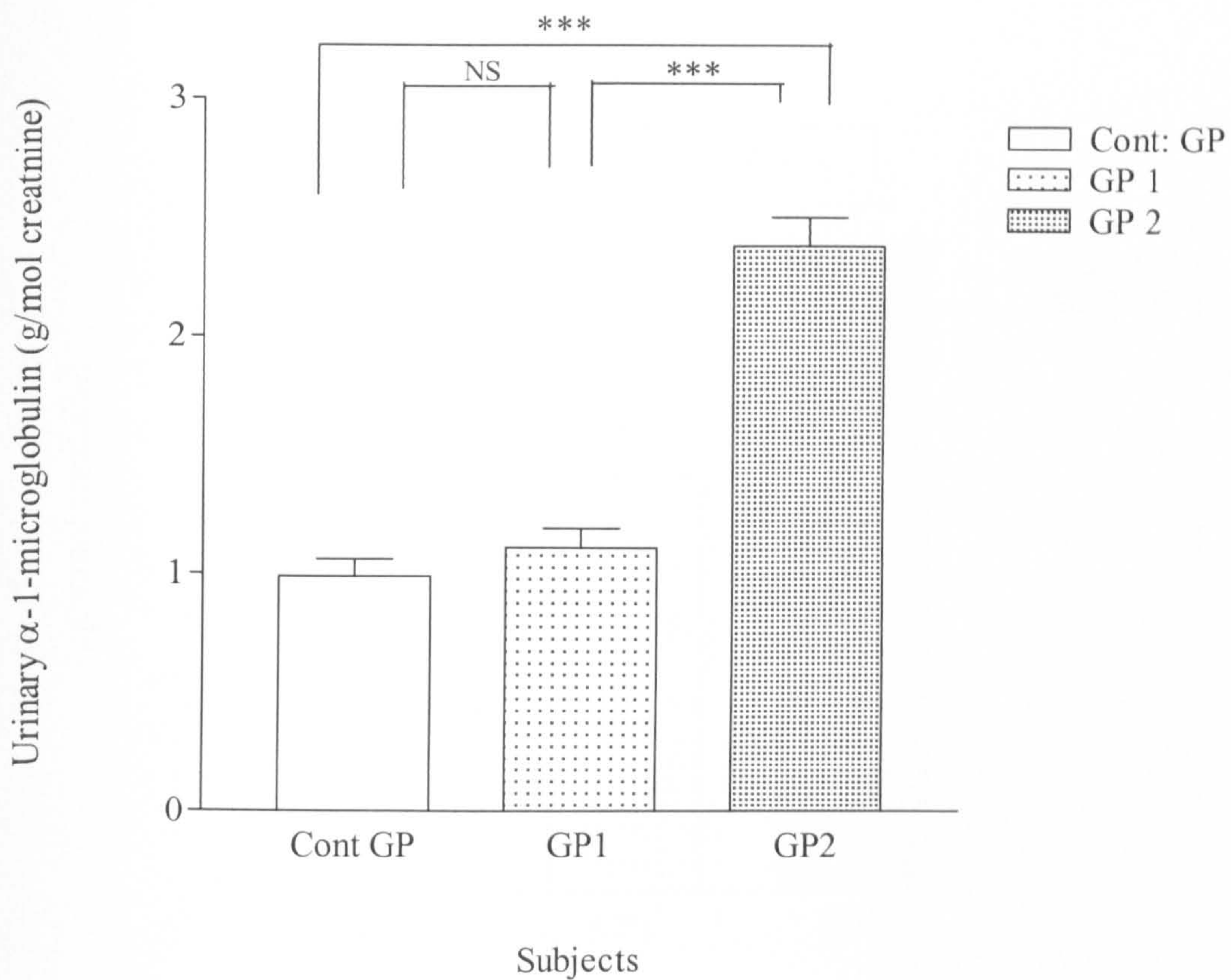


### **5.2.6 Urinary $\alpha$ 1-microglobulin (Figure 5.2.6)**

Increased urinary  $\alpha$ 1-microglobulin (g/mol creatinine) was observed in the established diabetic group (GP2) compared with values obtained from the both recently diagnosed diabetic group (GP2 vs GP1,  $p < 0.001$ ) and control group (GP2 vs Cont GP,  $p < 0.001$ ) subjects. In the recently diagnosed diabetic group,  $\alpha$ 1-microglobulin values were similar to those of control group subjects, and within the stated reference range ( $< 1.58$  g/mol creatinine). However, a 2-fold elevation  $\alpha$ 1-microglobulin values were observed in the established diabetic group compared to both the control and recently diagnosed diabetic group values.

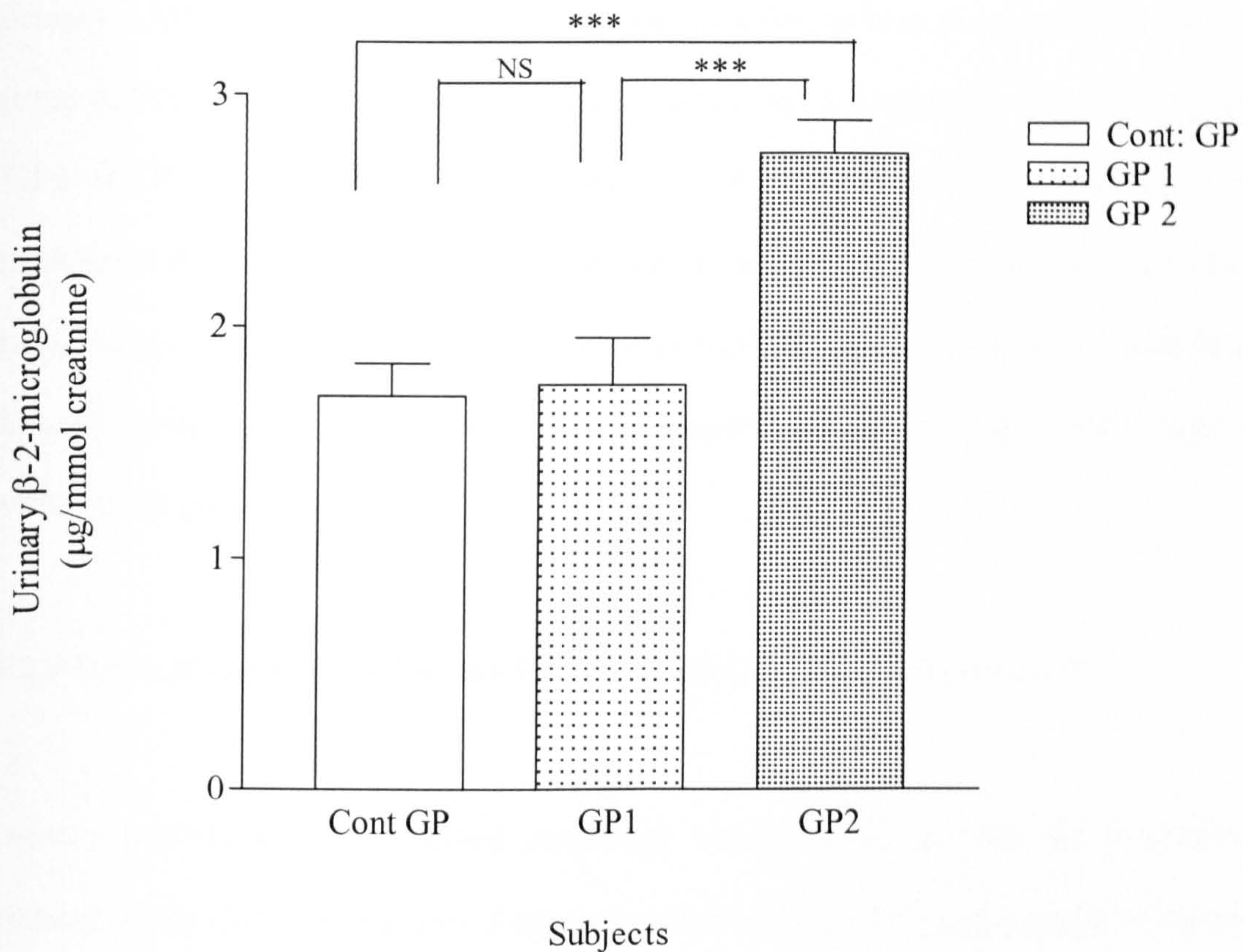
### **5.2.7 Urinary $\beta$ 2-microglobulin (Figure 5.2.7)**

Urinary  $\beta$ -2-microglobulin ( $\mu$ g/mmol creatinine) was also only significantly increased in the established diabetic group (GP2) compared with results from the recently diagnosed diabetic group (GP2 vs GP1,  $p < 0.001$ ) and control group (GP2 vs Cont GP,  $p < 0.001$ ) subjects.  $\beta$ 2-microglobulin levels were similar in both the control and recently diagnosed diabetic groups and within the stated reference range ( $0.8$ - $2.2$   $\mu$ g/mmol creatinine). A 1.5-fold elevation in  $\beta$ 2-microglobulin levels were observed in the established diabetic group when compared to values obtained from both the recently diagnosed diabetic group and control group subjects.



**Figure 5.2.6** Urine  $\alpha$ 1-microglobulin (g/mol creatinine) in control (Cont GP), GP1 and GP2 subjects. Results (Mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).





**Figure 5.2.7** Urine  $\beta$ 2-microglobulin ( $\mu\text{g}/\text{mmol creatinine}$ ) in control (Cont GP), GP1 and GP2 subjects. Results (Mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) performed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).

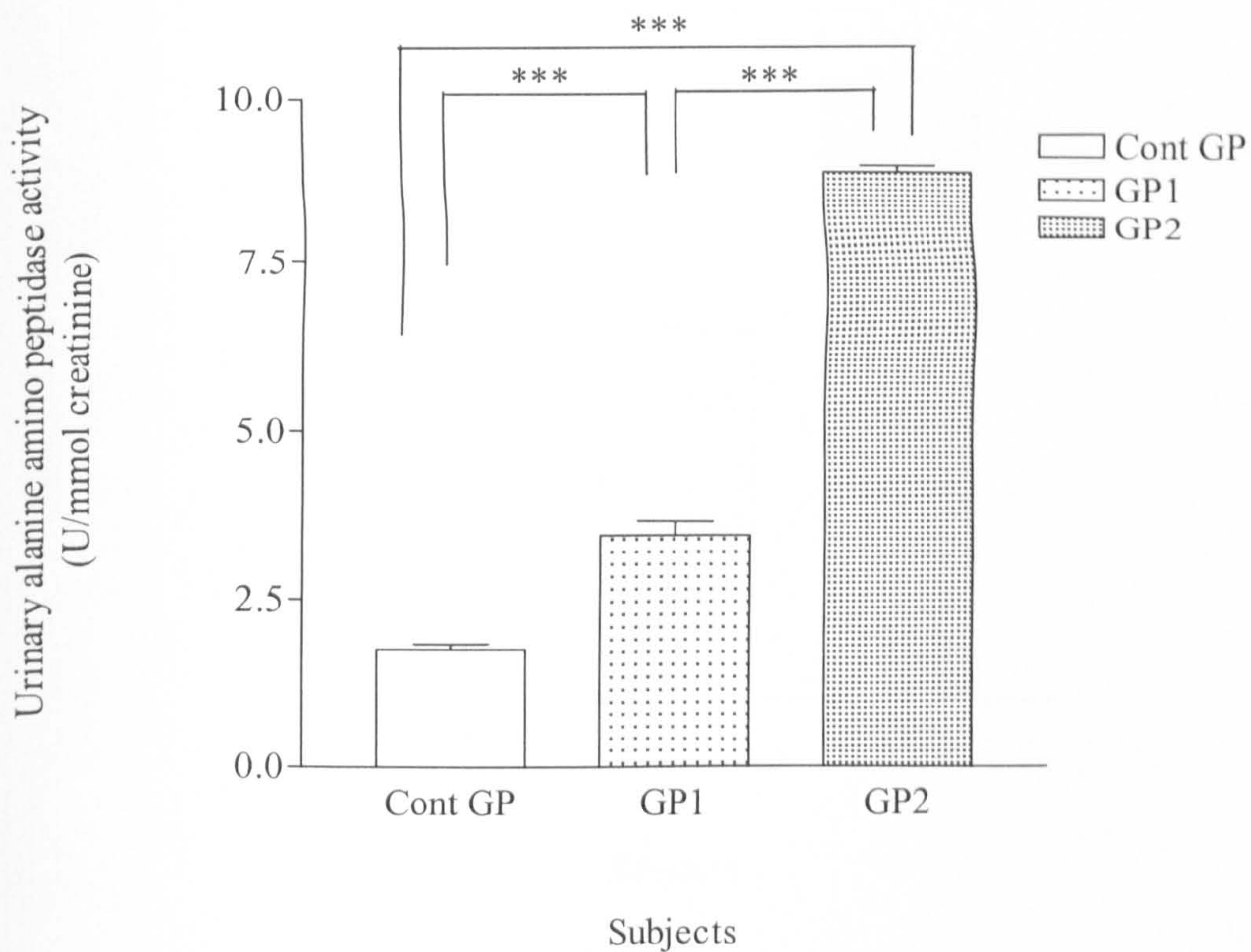


#### **5.2.8 Urine alanine amino peptidase (AAP) activity (Figure 5.2.8)**

Urinary AAP activity (U/mmol creatinine) was increased in both the established diabetic group (GP2) and recently diagnosed diabetic group (GP1) compared with control group (GP2 vs GP1 vs Cont GP,  $P < 0.001$ ) values. Highest AAP activities were observed in the established diabetic group compared with both recently diagnosed diabetic and control group values. The mean urinary AAP activities were increased 5-fold in the established diabetic group (GP2) and 3-fold in recently diagnosed diabetic group when compared with control group values.

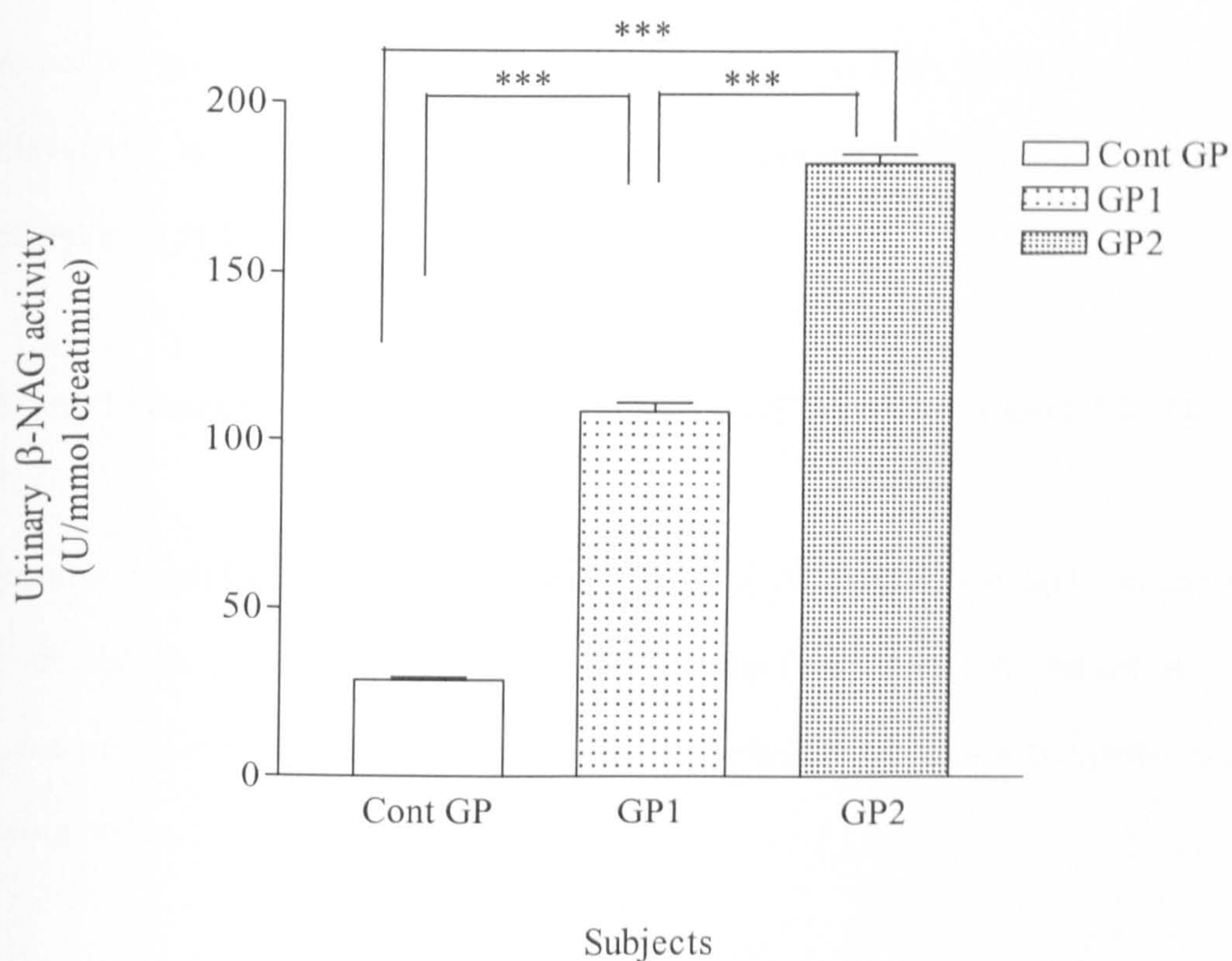
#### **5.2.9 Urine N-acetyl- $\beta$ -D-glucosaminidase ( $\beta$ -NAG) activity (Figure 5.2.9)**

Urinary  $\beta$ -NAG activity (U/mmol creatinine) was increased in both the established diabetic group (GP2) and recently diagnosed diabetic group (GP1) compared with control group (GP2 vs GP1 vs Cont GP  $P < 0.001$ ) values. The higher elevation of  $\beta$ -NAG activity was observed in the established diabetic group compared with both recently diagnosed diabetic group and control group. Control group,  $\beta$ -NAG activities were within the laboratory reference interval ( $< 35$  U/mmol creatinine). The mean urinary  $\beta$ -NAG activities were increased 5-fold in the established diabetic group and 3-fold in the recently diagnosed diabetic group when compared with control group values.



**Figure 5.2.8** Urinary AAP activities (U/mmol creatinine) in control (Cont GP), GP1 and GP2 subjects. Results (Mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferrionie's multiple comparison test. (\*\*\*,  $P < 0.001$ ).





**Figure 5.2.9** Urinary  $\beta$ -NAG activities (U/mmol creatinine) in control (Cont GP), GP1 and GP2 subjects. Results (Mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).

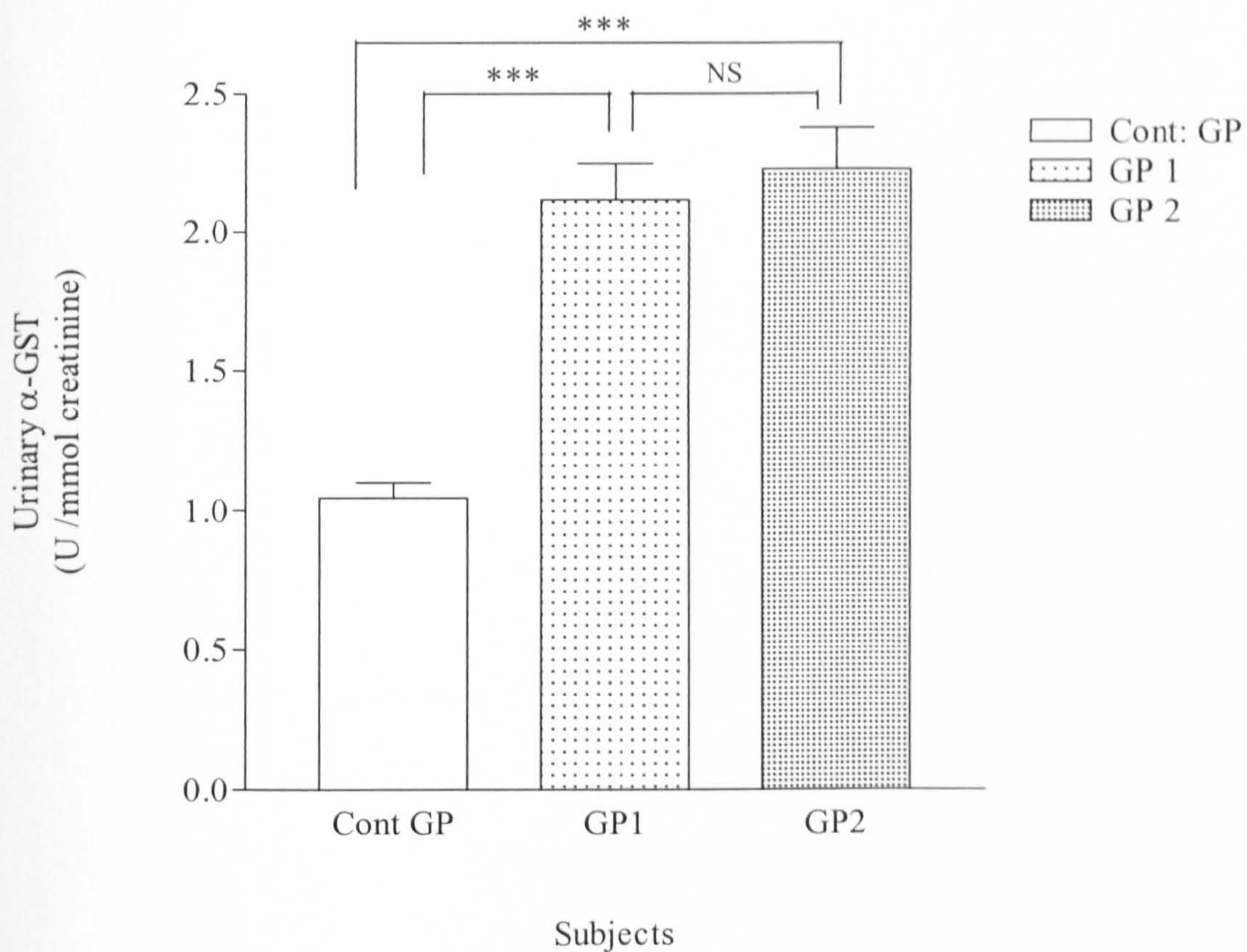


#### **5.2.10 Urinary alpha-glutathione-S-transferase ( $\alpha$ -GST) activity (Figure 5.2.10)**

Urine  $\alpha$ -GST activity (U/mmol creatinine) was significantly elevated in both the established diabetic group (GP2) and recently diagnosed diabetic group (GP1) compared to control group (GP2 vs Cont GP,  $P < 0.001$ , GP1 vs Cont GP,  $P < 0.001$ ) values. Similar elevations in activity were observed in both the recently diagnosed diabetic and established diabetic groups.

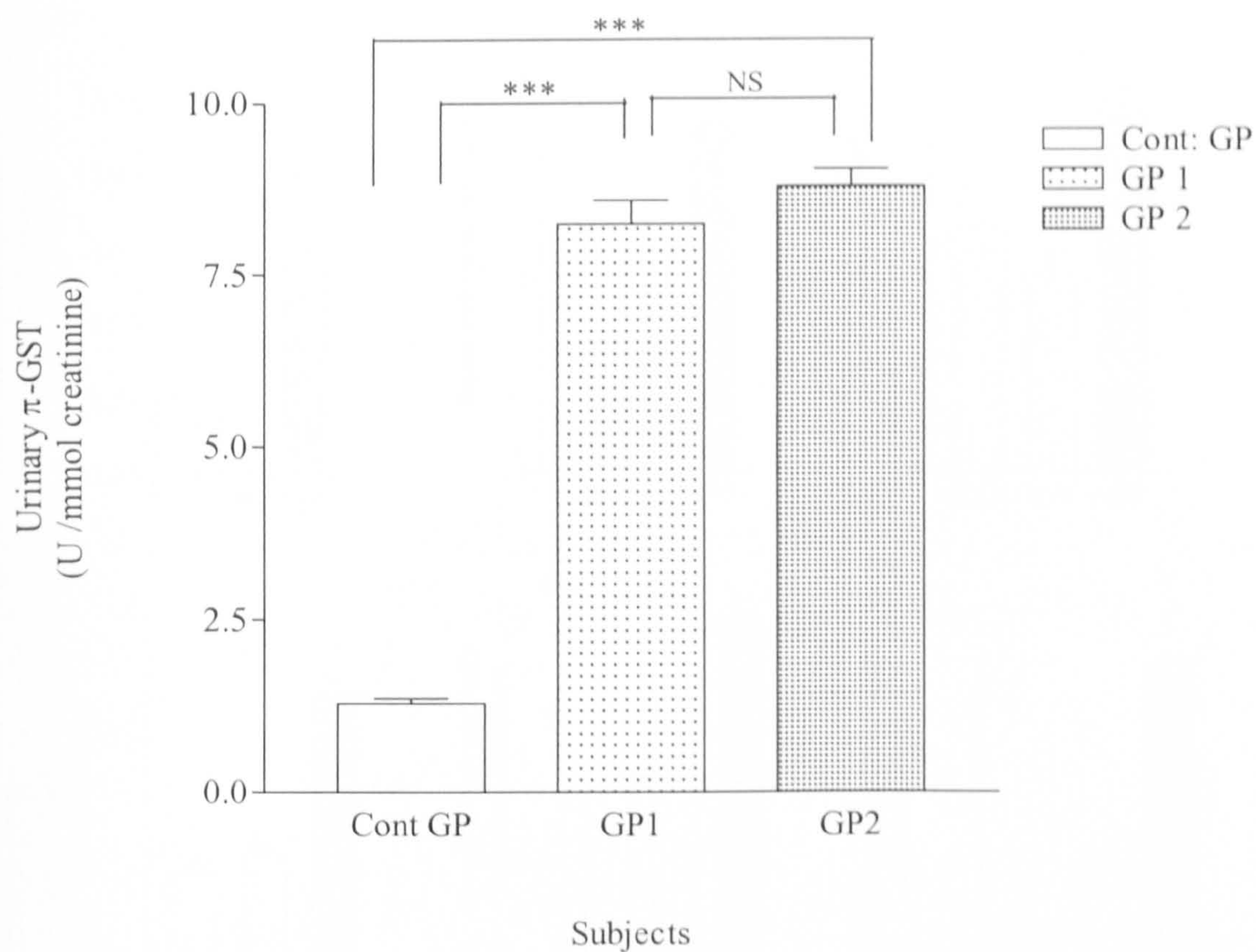
#### **5.2.11 Urinary pi-glutathione-S-transferase ( $\pi$ -GST) activity (Figure 5.2.11)**

Similar, significant increased urine  $\pi$ -GST activity (U/mmol creatinine) was observed in both diabetic groups compared with control group (both,  $P < 0.001$ ) values. A 7-fold increase in urine  $\pi$ -GST activities were noted in both diabetic groups compared to control group values.



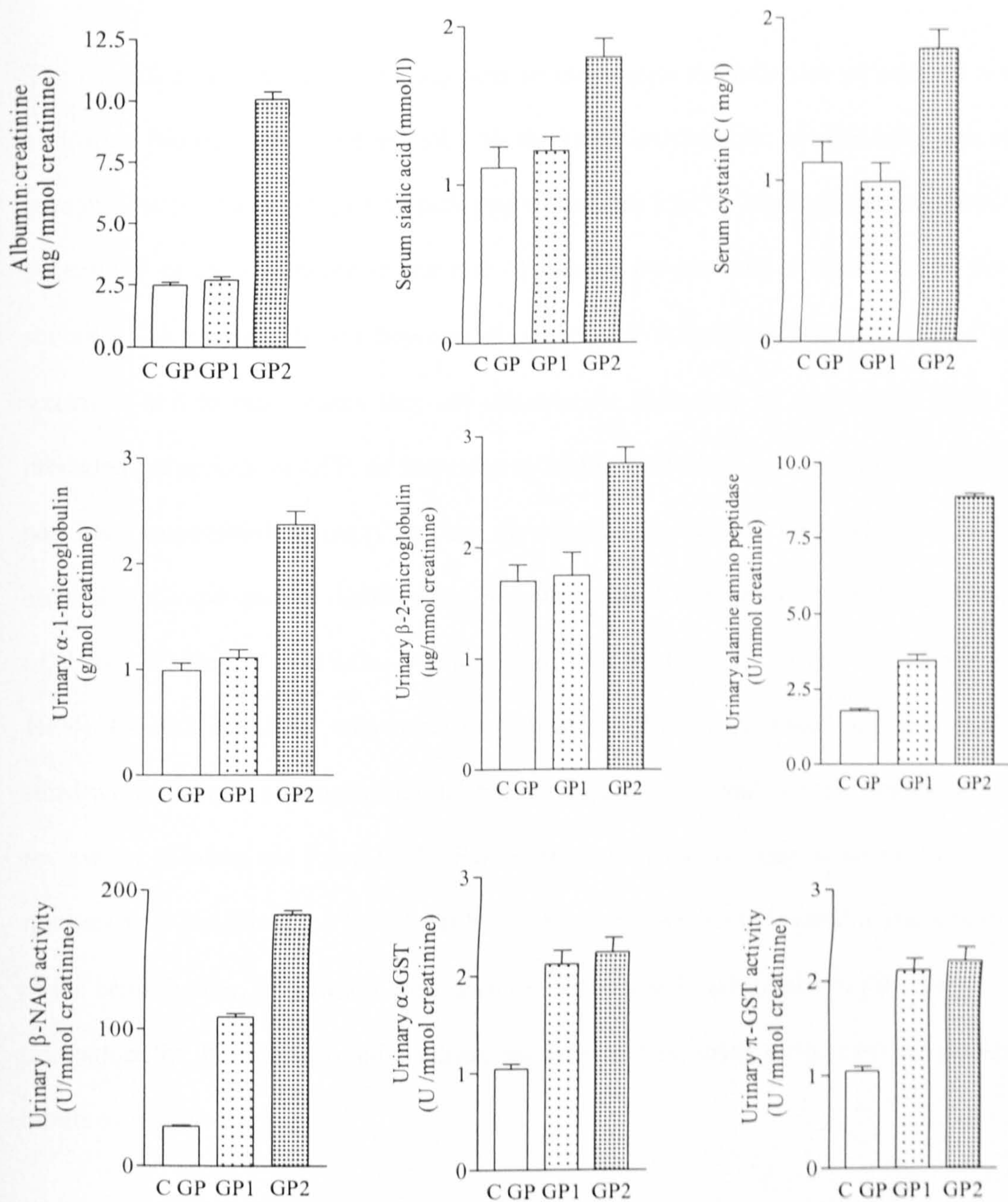
**Figure 5.2.10** Urine  $\alpha$ -GST level (U/mmol creatinine) in control (Cont GP), GP1 and GP2 subjects. Results (Mean  $\pm$  SEM) were compared using a one-way analysis of variance test (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).





**Figure 5.2.11** Urine  $\pi$ -GST (U/mmol creatinine) in control (Cont GP), GP1 and GP2 subjects. Results (Mean  $\pm$  SEM) were compared using a one-way analysis of variance (1-way ANOVA) followed by the Bonferroni's multiple comparison test. (\*\*\*,  $P < 0.001$ , NS: not significant).





**Figure 5.2.12** Summary of the renal biomarkers. C GP: control group, GP1: recently diagnosed diabetic group, GP2: established diabetic group.



### 5.3 Discussion

The overall aim of the present study was to investigate the behavior of selected serum and urine biomarkers reflecting insult to the renal architecture. In particular, the early recognition of renal insult in a diabetic population can lead to improved management and potentially either a slowing in the rate of disease progression or reduction in disease severity. The markers chosen, however in previous investigations have been shown to be sensitive, and in many cases they are site-specific indicators of early renal insult that preceded reductions in GFR or increases in serum creatinine concentration. Enzymuria has been quantitated in many studies on either drug-induced nephrotoxicity in both clinical and experimental diabetes and in both medical and surgical studies (Whiting *et al*, 1980, Whiting *et al*, 1983a; Whiting *et al*, 1983b; UKPDS, 1993; Weitgasser *et al*, 1999).  $\beta$ -NAG and AAP enzymuria have previously been increased and shown to be sensitive indicators of renal proximal tubular insult in several disease processes (for a review see Whiting and Price 2001). Furthermore, they can be considered as site-specific markers with NAG being a lysosomal hydrolase and AAP being located in mitochondria of the brush border. In addition, measurement of the small molecular weight proteins,  $\alpha$ 1-microglobulin,  $\beta$ 2-microglobulin, allows the transport of small protein by the proximal tubule to be assessed.

Glutathione-S-transferase (GST) activity, both  $\alpha$  and  $\pi$  isoenzymes has recently been proposed as markers of proximal and distal tubular integrity respectively (Sundberg, 1994; Fujita, 2001; Maxwell and Gordon, 2002). In addition, while serum creatinine concentrations are a standard and commonly used estimate of GFR, cystatin C, a new

sensitive biochemical marker, has also been demonstrated recently to offer advantages in the evaluation of glomerular function, particularly in diabetes mellitus. The results of the present study clearly demonstrate altered renal glomerular and tubular integrity and function that predated alterations in serum creatinine concentration that remained within the reference range in all three experiment groups.

Initially, the association between microalbuminuria, serum creatinine, sialic acid and cystatin C concentrations in diabetic states was investigated. Increased albumin:creatinine ratio, sialic acid and cystatin C concentrations were observed in the established diabetic group compared to those from the recently diagnosed diabetic group and control group subjects (Figure 5.2.2, 5.2.4, 5.2.5). Consequently, this observation confirmed that the established diabetic group is 'microalbuminuric' whereas the recently diagnosed diabetic group is 'normoalbuminuric'. However, serum creatinine concentrations in both groups were within the standard reference for the laboratory (Section 5.2.1) suggesting that increased serum creatinine concentration is a late manifestation of diabetic nephropathy; GFR is only reduced when 50% of the nephrons cease to function and is at best an insensitive measure of glomerular function (Whiting and Price, 2001).

The established diabetic group, demonstrated increased fasting blood glucose concentration, fructosamine and glycated haemoglobin (HbA<sub>1c</sub>) levels (as described in the Chapter 3) that were associated with an increased albumin:creatinine ratio. (increased HbA<sub>1c</sub> levels directly correlated with microalbuminuria levels in the established diabetic group subjects as shown in Figure 5.2.3). Consequently, results of the present study are



consistent with previous studies (DCCT, 1993; UKPDS, 1998; ADA, 2000) that demonstrated persistent hyperglycaemia and poor metabolic control significantly increased the risk for the development of microalbuminuria.

Furthermore, protein glycation was associated with the changes in glomerular permeability that may be a determining feature in the loss of negative charge from the GBM; leading to less repulsion of anionic proteins, and consequent altered glomerular permeability in subjects with diabetes (Ward, 1995). Sialic acid are found in the GBM glycoproteins associated with heparin sulfate proteoglycans, which contribute to the negative charge/size selectivity of the GBM. Increased serum sialic acid concentrations have been observed previously to be associated with diabetic retinopathy and nephropathy (Sillanaukee *et al*, 1999). Consequently, increased microalbuminuria (albumin:creatinine ratio) and serum sialic acid content may be explained, at least in part, by the loss of the glomerular polyanion, associated decrease in negative charge and biochemical alterations to the glomerular capillary wall (Yokoyama *et al*, 1996) in subjects with persistent hyperglycaemia. However, the mechanism of increased serum sialic acid in subjects with diabetes and diabetic nephropathy still remain unclear (Sillanaukee *et al*, 1999).

The GFR still provides the best overall estimation of renal function and serum cystatin C, a protease inhibitor, has been suggested as a new sensitive biochemical marker of GFR (Newman *et al*, 1995; Stabuc *et al*, 2000). Consequently, the observation of normal serum creatinine concentration but increased microalbuminuria, sialic acid and cystatin C

concentrations in the established diabetic group further suggested that the former analyte i.e. serum creatinine is of limited value in the early detection of renal insult because of its poor sensitivity to early nephron dysfunction (Perrone *et al*, 1992; Newman *et al*, 1995). Consequently, the present study supports the notion that cystatin C is a useful and sensitive biochemical marker for the overall estimation of renal function (Stabuc *et al*, 2000) in established diabetic subjects.

Kidney disease can be differentiated by the estimation of selected proteins in urine. Consequently, estimation of renal tubular function and integrity may provide an early indication of renal insult and potentially identify those at risk of developing pre-clinical kidney dysfunction (Whiting and Price, 2001). A lesion of the renal tubular apparatus is characterized by a reduced reabsorption of low molecular weight proteins, such as  $\alpha$ 1-microglobulin and/or  $\beta$ 2-microglobulin, which are completely filtered at the glomerular and 99% reabsorbed by the proximal tubules. Significant elevation of both urinary  $\alpha$ 1-microglobulin (Figure 5.2.6) and  $\beta$ 2-microglobulin (Figure 5.2.7) concentrations were observed in the microalbuminuric group subjects compared to both normoalbuminuric and control group subjects suggesting renal tubular insult reduced protein absorptive capacity. The urinary concentration of proteins will depend on the filtered load i.e. glomerular permeability, and on the proximal tubular reabsorptive process utilizing clathrin coated pits in the brush border of proximal tubular cells (Newman and Price, 1999). Consequently, the  $\alpha$ 1-microglobulin and  $\beta$ 2-microglobulin appear in significantly higher amounts in urine as a result of decreased tubular reabsorption capacity and therefore will provide evidence for the so-called tubular proteinuria and loss

of function in the proximal tubular (Jung and Jung, 1994; Newman and Price, 1999). Furthermore, insult of the tubular epithelial cells will result not only functional and biochemical changes but also ultimately in structural changes with increased cellular function and cell lysis resulting in the release of brush border enzymes such as AAP (Jung *et al*, 1988; Jung, 1994). The increased AAP activity observed in both microalbuminuric group and normoalbuminuric group compared to control group subjects (Figure 5.2.8) suggests that AAP is an indicator of early tubular changes even when the glomerular filtration and renal plasma flow are still within the 'normal' limits or marginally deteriorated although without histological changes (Jung, 1994; Holdt-Lehmann *et al*, 2000).

Evidence suggests that urinary enzyme excretion is increased in the earliest stage of diabetic nephropathy (Jung *et al*, 1988; Whiting *et al* 1979; Price, 1979).  $\beta$ -NAG a lysosomal proximal tubular cell enzyme increase was found to be associated with early diabetic nephropathy and poor long-term glycaemic control (Watts *et al*, 1988). In the present study,  $\beta$ -NAG enzyme activities were elevated in both microalbuminuric group and normoalbuminuric subjects compared to control group subjects, with the highest activity been observed in the established diabetic group (microalbuminuric) subjects (Figure 5.2.9). Elevated NAG activities were directly associated with HbA<sub>1c</sub> levels in both diabetic groups. Furthermore, the proximal tubules are the most metabolically active renal segment and potentially vulnerable to damage and evidence suggests that elevated  $\beta$ -NAG activity may be consequences of hyperglycaemia and increased polyol pathway activity associated with proximal tubular insult resulting in abnormal urinary enzyme



excretion (Ishii *et al*, 2001) may all be involved. Evidence suggests that the increased  $\beta$ -NAG enzymuria increases in diabetic state (Whiting *et al*, 1979; Price, 1992; UKPDS, 1993) occurs prior to increased glomerular permeability as the two parameters measure different areas of renal integrity i.e. proximal tubular insult and glomerular permeability, respectively (Martin *et al*, 1990). This suggestion is further supported by the observation that renal tubular insult may precedes increased glomerular permeability and  $\beta$ -NAG enzymeuria is an early indicator of renal insult (Whiting and Price, 2001) in the progression of early diabetic nephropathy and incipient tubulopathy (Holdt-Lehmann *et al*, 2000).

Skrha and Hilgertova, (1999) recently demonstrated that subjects with diabetes and enhanced oxidative stress had higher serum NAG activities before the development of microalbuminuria. A significant positive correlation between serum NAG activities and plasma MDA concentrations was observed in this study suggesting that oxidative stress may precipitate tissue damage and the autophagic response to this damage will result in lysosomal proliferation and increased NAG enzyme release. A decreased NAG activity observed after treatment with vitamin E ( $\alpha$ -tocopherol) may further support the role of free radical mediated oxidative stress in the tissue insult that resulted in the increased NAG enzymuria (Skrha and Hilgertova, 1999). The results of the present study, which clearly demonstrated that direct association between increased urine, NAG activities, and TBARS (as described in Chapter 4) in both the established diabetic group (microalbuminuric) and recently diagnosed diabetic group (normoalbuminuric) subjects is consistent with this suggestion. This evident suggests that oxidative damage and renal

tubular insult may be associated with the onset of microalbuminuria. Furthermore, in an experimental animal model, those animals that exhibited albuminuria, had significantly higher levels of lipid peroxides in plasma, urine and renal proximal tubules, suggesting that increased oxidative stress in the diabetic kidney and lipid peroxidation is an important biological consequence of the free radical mediated oxidative process (Yaqoob *et al*, 1994; Koya *et al*, 1997; Koya *et al*, 2003).

The toxicity of products of lipid peroxidation in a cell is reduced in part by GSH-Px and in part by other intracellular phase II defensive systems, for example, these involving glutathione-S-transferases (GSTs); although, both enzymes are necessary for the detoxification of the products of lipid peroxidation. While GSTs are cytosolic enzymes the alpha isoenzyme ( $\alpha$ GST) is present only in renal proximal tubular cells, whereas the pi ( $\pi$ GST) isoenzyme is confined to renal distal tubular cells as shown by immunohistochemical studies (Harrison *et al*, 1989; Sundberg *et al*, 1994; Branten *et al*, 2000). Furthermore, both  $\alpha$ GST and  $\pi$ GST activities were increased in the microalbuminuric group subjects compared to both normoalbuminuric and control group subjects (Figure 5.2.10, Figure 5.2.11). This suggests that while increased  $\alpha$ GST levels were associated with proximal tubular insult and brush border damage (Branten *et al*, 2000) the increased  $\pi$ GST levels were consistent distal tubular insult. The increased activities were also associated with glomerular permeability, manifested by increased microalbuminuria. A similar study performed by Branten *et al*, (2000) also demonstrated a direct association between  $\pi$ GST levels and increased serum creatinine and proteinuria and also suggested that a distal tubular insult was associated with glomerular

permeability. Furthermore, Fujita *et al*, (2001) demonstrated that  $\alpha$ GST was increased in the proximal tubules, before the manifestation of the typical diabetic histopathological changes in the glomeruli of diabetic animals suggesting that increased expression of GST has the potential to be one of the earliest physiological adaptations to hyperglycaemia or early renal insult. Consequently, the identification of those changes, particularly in the early stages of diabetes, may well contribute to the elucidation of the basic mechanisms of diabetic nephropathy and permit a better understanding of the underlying pathophysiology (Fujita *et al*, 2001).

Free radical mediated oxidative stress triggered by hyperglycaemia is thought to be a significant determinant of such renal tubular changes. The observed increased activities of GSTs may well be a protective response of renal tubular cells to the oxidative damage or other toxic effects of glucose. Furthermore, these toxic effects of glucose may ultimately contribute insult to both proximal and distal renal tubular system (Fujita *et al*, 2001). However, the underlying mechanism that lead to an association of GST with increased glucose-induced lipid peroxidation and enhanced oxidative stress in diabetes in general and diabetic nephropathy in particular still need to be clearly defined. Other evidence also suggest that the formation of lipid peroxides could be a major triggering factor for apoptosis and enhanced oxidative stress in diabetic kidney could therefore induce apoptosis, which would also contribute to the development of diabetic nephropathy (Zhang *et al*, 1997; Kannan and Jain, 2000).



## **CHAPTER SIX**

### **GENERAL DISCUSSION**

## 6. General discussion

One of the main aims of the present studies was to investigate if the generation of free radicals and lipid peroxides, associated dyslipidaemia and renal insult was temporally related to the presence of hyperglycaemia. Consequently, length of disease duration rather than the type of diabetes present, was a major consideration in selecting study populations. However, this study design has the limitation that both diabetic study groups contained a mixture of Type 1 and Type 2 diabetic subjects. This limitation is due to the heterogeneous nature of the type 2 diabetic state.

The present study has clearly demonstrated that persistent hyperglycaemia was the major determinant that linked altered glucose and lipid metabolism with the generation of free radicals, resulting in increased lipid peroxidation and depletion of antioxidant inhibitory and scavenger mechanisms and thereby enhanced oxidative stress. An association between persistent hyperglycaemia-induced enhanced free radical mediated oxidative stress and profound early renal functional, and biochemical changes predating early diabetic nephropathy was also observed. In particular, the present studies have also demonstrated altered glomerular function in the presence of both proximal and surprisingly, distal renal tubular insult.

## 6.1 Altered glucose, lipid metabolism and generation of free radicals

Previous clinical and experimental studies (Mauer *et al*, 1975; Weil *et al*, 1975; DCCT, 1993; UKPDS, 1998; Stratton *et al*, 2000) have clearly demonstrated that hyperglycaemia and consequent poor glycaemic control is the main metabolic determinant associated with irreversible kidney damage in diabetes. The present study has confirmed and extended these earlier observations and a strong association between persistent hyperglycaemia and altered metabolic parameters such as glycated haemoglobin, fructosamine and lipoproteins in a cohort of diabetic subjects was also noted. Consequently, observations from the present study are consistent with the suggestion that both poor glycaemic and metabolic control may contribute to the progression and the development of diabetic nephropathy. Conversely, improved glycaemic control effectively delays the onset and retards the progression of diabetic nephropathy (UKPDS, 1998). Consequently, these observations provide impressive clinical evidence that hyperglycaemia *per se* is the major pathogenic factor in the development and onset of diabetic complications in general and diabetic nephropathy in particular.

Amongst the irreversible changes that occur as a direct result of persistent hyperglycaemia are the formation of AGEs following protein glycation (Bucala *et al*, 1993; Brownlee, 1995; Vlassara, 1995; Schmidt *et al*, 1995; Vlassara, 1996, Singh *et al*, 2001). In the diabetic state the importance of these processes is manifest in two essential factors namely the changes in protein structure and function as a result of glycation and the use of glycated protein levels as a indicator of chronic/long term glycaemic control. A



classical example of non-enzymatic glycation is the formation of glycated haemoglobin, or, more precisely, HbA<sub>1c</sub> as measured in the present study.

In the established diabetic group increased fasting plasma glucose and fructosamine concentrations were directly associated with elevated HbA<sub>1c</sub>. Furthermore, chronic or persistent hyperglycaemia in diabetes has been well established to accelerate synthesis and tissue deposition of AGEs, an abnormality contributing to the pathogenesis of co-morbid complications. (West, 2000). A number of AGEs such as *N*<sup>ε</sup>-[carboxymethyl]-lysine (CML), pyralline and pentosidine have been identified in the renal tissue of diabetics with or without ESRD, with AGEs accumulation increasing with the severity of diabetic nephropathy (Sugiyama *et al*, 1996).

The elevated fructosamine and glycated haemoglobin levels associated with increased lipid peroxidation and reduced antioxidants and associated defence mechanisms noted in the established diabetic group implicates AGEs as a source of free radicals leading to enhanced oxidative stress and subsequent renal insult as suggested in Fig 1.3.7.2 in the introduction. However, although AGEs were not measured in the present studies due to resource and time limitations, their quantitation would have allowed a more detailed approach to demonstrating their role, as one end product of free radical activity in the pathogenesis of diabetic nephropathy. Furthermore, the generation of AGEs products, linked to the molecular mechanisms underlying the pathogenesis of renal dysfunction via ligation of AGEs receptors in renal tissue and the activation of the DAG-PKC pathway (Hunt *et al*, 1988; Inoguchi *et al*, 1992), may be just one pathway involved in the

multifactoral process precipitating changes in both GFR and renal tubular function. This may, of course ultimately also result in as increased susceptibility to free radical mediated oxidative stress and vascular dysfunction, which may be important in the early developmental stages of diabetic complications (Lee and Chung, 1999; Nakamura *et al*, 2000).

Many studies have demonstrated a direct link between hyperglycaemia and increased total cholesterol, triglycerides, LDL, and decreased HDL concentrations as a consequence of diabetic dyslipidaemia (Carvajal *et al*, 1983; Abbate and Brunzell, 1990; Tolins *et al*, 1992; Taskinen, 1987; Taskinen, 1996; Betteridge, 1997). The present study also clearly demonstrated that increased fasting glucose concentrations, fructosamine and HbA<sub>1c</sub> levels were observed in the presence of increased total cholesterol, triglycerides, LDL, and decreased HDL concentrations in established diabetes. These observations are consistent with those of Wanner *et al*, (2001) that suggested a direct association between poor glycaemic and metabolic control and diabetic dyslipidaemia. Furthermore, in subjects with diabetic nephropathy, hyperlipidaemia has been identified as a major risk factor for the rapid decline of GFR and increased mortality.

Recently, high triglycerides and low HDL cholesterol concentrations have been identified as strong predictors of a more rapid progression of microalbuminuria in type 2 diabetic subjects with well controlled blood pressure (Wanner *et al*, 2001). However, when glycaemic control is poor, total serum cholesterol concentrations in diabetes can be significantly enhanced due to the additional accumulation of LDL (Taskinen *et al*, 1996), results similar to those observed in the established diabetic group investigated in the

present study. Furthermore, diabetic subjects with good or reasonable glycaemic control still tended to demonstrate LDL concentrations within the laboratory reference range. Kramer-Guth *et al*, 1996; Kramer-Guth *et al*, 1997, an observation similar to that observed in the recently diagnosed diabetic group in the present study. Overall, the results of the present study in which persistent hyperglycaemia was associated with increased protein glycation and dyslipidaemia suggests a central role for the latter, not only in diabetic atherogenesis but also in the pathogenesis diabetic nephropathy (Bucala *et al*, 1993; Vlassara, 1995, Galle *et al*, 1999).

## **6.2. Increased lipid peroxidation, depletion of antioxidants and enhanced oxidative stress**

Previous clinical and experimental studies have demonstrated enhanced lipid peroxidation in subjects with diabetes (Sato *et al*, 1979; Armstrong and Al-Awadi, 1991; Armstrong *et al*, 1992; Akkus *et al*, 1996; Santini *et al*, 1997, Hartnett *et al*, 2000). The direct association observed between increased fasting glucose concentrations and TBARS excretion in both diabetic groups in general, but the established diabetic group in particular, is consistent with hyperglycaemia-induced enhanced lipid peroxidation in diabetics, results consistent with those of earlier clinical and experimental studies.

However, although in the present studies a trend towards increased lipid peroxidation associated with increased total cholesterol, triglycerides, LDL, and decreased HDL concentrations was observed in the established diabetic group, this did not achieve



statistical significance. Although the reason for this is not clear it may be that the process of peroxidation does not depend on lipoproteins levels and the increased total amount of circulating lipid plays an additional and exacerbating role in the enhancing this process in the presence of persistent hyperglycaemia as suggested by both Griesmacher *et al*, (1994) and Mooradian, (1991). Furthermore, although it is now well established that AGEs, lipid-advanced glycation and lipid oxidation processes generate free radicals and initiate lipid peroxidation in diabetic subjects with inadequate glycaemic and metabolic control (Bucala *et al*, 1993, Halliwell and Gutteridge, 1998) the control mechanisms remain unclear.

Many studies have previously demonstrated significant depletion of antioxidants such as reduced plasma total antioxidant status (Sundharam *et al*, 1996; Maxwell *et al*, 1997), plasma vitamin E (Salonen *et al*, 1995, Reavan *et al*, 1995), both erythrocyte and plasma glutathione peroxidase (Mukherjee *et al*, 1998; Aaseth and Stoa-Birketvedt, 2000; Varvarovska *et al*, 2003) activities, and plasma selenium content (Schienger *et al*, 1988, Faure *et al*, 1993; Bennefont-Rousselot *et al*, 1997) in the diabetic state. Results from the present study also clearly demonstrated significantly decreased plasma total antioxidant status, plasma vitamin E content, both erythrocyte and plasma glutathione peroxidase activities, and plasma selenium content but only in the established diabetic group. Furthermore, significant correlations between HbA<sub>1c</sub> and TBARS ( $r^2 = 0.894$ ,  $P < 0.001$ ), HbA<sub>1c</sub> and vitamin E ( $r^2 = 0.367$ ,  $P < 0.001$ ), and TBARS and vitamin E levels ( $r^2 = 0.896$ ,  $P < 0.0001$ ) were also noted in the present studies. This suggests that HbA<sub>1c</sub> and glycaemic control are linked to both the rate of lipid peroxidation and depletion of

extracellular antioxidant scavengers and the presence of enhanced oxidative stress in the established diabetic group, consistent with the suggestion of Jain *et al*, (2000). The study of Armstrong *et al*, (1992) has suggested that reduced free radical production as a result of improved metabolic control would lead to reduced consumption of chain-breaking antioxidants and that could eventually therefore, contribute to the improved antioxidant status and reduced lipid peroxidation, again adding weight to this suggestion. Further evidence supporting the presence of enhanced oxidative stress in the diabetic state has also been provided by animal studies (Ha and Kim, 1999) and by vitamin E dietary supplementation (Koya *et al*, 1997; Ha and Kim, 1999, Koya *et al* 2003). Furthermore, vitamin E supplementation studies have recently revealed that increased cellular glutathione levels, decrease HbA<sub>1c</sub> levels and MDA concentrations were associated with a reduced incidence of vascular disease in poorly control diabetics (Jain *et al*, 2000).

Glutathione peroxidases play a central role in the defence mechanisms dealing with lipid hydroperoxides, utilising glutathione and NADPH in the process to yield hydroxyl compounds, and hence in preventing the development of oxidative stress. In the present study plasma and erythrocyte glutathione peroxidase activities were decreased in the recently diagnosed diabetic group and further decreased in the established diabetics. Plasma glutathione peroxidase has been shown to originate from the renal proximal tubular cells and the parietal cells of the Bowman's capsule and is released into the blood via the efferent and peritubular capillaries. The proximal tubules are also the site of synthesis of selenoproteins which could explain in part the relationship between plasma glutathione peroxidase activity and plasma selenium concentrations, a similar observation

to that of Bennefont-Rousselot *et al*, (1997). In this regard, the observations of Brigellius-Flohe, (1999) could also be particularly relevant in that his studies demonstrated that a transient selenium deficiency, precipitating a rapid impairment of plasma glutathione peroxidase activity, was associated with a high index of both micro and macroangiopathy in diabetic subjects but without evidence of overt nephropathy being noted.

In normal conditions, cellular defence mechanisms are sufficient to counteract the effects of free radicals but in the diabetic state they may be overwhelmed because of increased oxidative stress. The present study has clearly demonstrated that lipid peroxidation is increased in the presence of persistent hyperglycaemia which, along with the observations of reduced counteracting defence mechanisms, is consistent with the presence of profound oxidative stress being present in the diabetic state. This in turn is associated with diabetic complications in general and diabetic nephropathy in particular characterised by structural, functional and biochemical changes in diabetic kidney (Douillet *et al*, 1999).

### **6.3 Renal glomerular and tubular changes in the diabetic state**

The difficulties that lie in the early diagnosis of diabetic nephropathy lie predominantly in the natural history of the disease. Significant structural changes, particularly thickening of glomerular basement membrane and mesangial expansion, occur only after several years of diabetes (Mogensen *et al*, 1984). Indeed, the early stages of the disease development are clinically silent and detected at present only by measurements of



microalbuminuria (Mogensen *et al*, 1984). Furthermore, serum creatinine measurements used as an indication of GFR are at best insensitive and only become elevated once less than 50% of nephrons remain functional. The glomerulus also demonstrates both a charge and size selectivity during filtration the former due to the so called glomerular polyanion consisting predominantly of heparin sulphate, sialic acid and other proteoglycans (Mayers *et al*, 1982). GFR begins to decline as the glomerular filtration barrier loses its charge and/or size selectivity resulting in an increasing magnitude of proteinuria and renal dysfunction. Consequently, the results of the present study which show reduced cystatin C levels, a new biomarker for the measurement of GFR (Jung and Jung, 1994; Newman *et al*, 1995; Piwowar *et al*, 1999; Stabuc *et al*, 2000; Page *et al*, 2000) in the presence of 'normal' serum creatinine concentrations may provide a more sensitive indication of glomerular function in the diabetic state. In addition, the present study has also clearly demonstrated that increased microalbuminuria was significantly associated with elevated serum sialic acid concentrations in the established diabetic group subjects. Consequently, circulating cystatin C and sialic acid concentrations and quantitation of microalbuminuria may provide a more effective biochemical assessment of glomerular function in the diabetic state than serum creatinine and microalbuminuria alone as a predictor for incipient diabetic nephropathy.

In the diabetic state, while glomerular disease is characterized by increased albumin excretion, manifestation of tubulo-interstitial damage is much more difficult to identify until GFR is reduced by at least 50%. Consequently, a wide range of new biochemical markers reflecting both early renal damage and site-specificity within the kidney has been

developed. These include specific low molecular proteins such as  $\alpha_1$ -microglobulin and  $\beta_2$ -microglobulin, urinary enzymes such as  $\beta$ -NAG, AAP and glutathione S transferases (for a review see Whiting and Price, 2001).

The present study demonstrated a significant association between microalbuminuria and low molecular weight proteinuria (LMWP,  $\alpha_1$ -microglobulin and  $\beta_2$ -microglobulin) in the established diabetic group subjects. Both increased glomerular permeability and proximal tubular absorptive dysfunction or the inability of the proximal renal tubule to reabsorb excess small molecular weight proteins could explain this observation. The observation that the recently diagnosed diabetic group demonstrated reference range values for both LMWP and microalbuminuria could suggest either insufficient diabetes related renal insult was present or the mechanisms underlying tubular reabsorption of filtered proteins are less sensitive and primarily not involved in the development of incipient diabetic nephropathy.

However, increased AAP and  $\beta$ -NAG-enzymuria were significantly increased in both diabetic groups suggesting that hyperglycaemia, both early and persistent, is associated with both brush border and proximal tubular cell insult. This observation is similar to those of other studies (Whiting *et al*, 1980; Whiting *et al*, 1983a,b; Kordonouri *et al*, 1998). Consequently, AAP and NAG-enzymuria, a sensitive indicator of proximal tubular insult, are of use in the recognition of those diabetic subjects predisposed to diabetic nephropathy, although a direct effect of hyperglycaemia, linked to polyol pathway activity, on lysosomal function cannot be excluded (Ishii *et al*, 2001).

In the present study, both the excretion of  $\alpha$ -GST and that of  $\pi$ -GST were also increased in both recent onset and established diabetic subjects. As these particular enzymes are primarily located in the proximal and both distal tubules and collecting ducts, respectively (Harrison *et al*, 1989) this suggests insult at both these intrarenal sites. Moreover, a trend towards higher activities of both isoenzymes was observed in the established diabetic subjects with early diabetic nephropathy and their use as an adjunct for recognition of early renal insult in diabetic subjects deserves further attention.

#### **6.4 The presence of oxidative stress in the diabetic state**

There is now appreciable evidence that an oxidative injury occurs in the diabetic state which is in itself an important factor in the development of associated early renal insult and subsequent dysfunction (Kakkar *et al*, 1997; Arima and Ito, 2003). For example, microalbuminuria, a marker of glomerular insult, higher levels of lipid peroxides and decreased antioxidants in renal proximal tubules have all been causally related, supporting the concept of a hyperglycaemia-induced increased oxidative stress in diabetic nephropathy (Asayama *et al*, 1993; Ha and Kim, 1995; Sundaram *et al*, 1996; Kakkar *et al*, 1997; Ha and Kim, 1999; Koya *et al*, 2003).

The present study clearly demonstrated that recently diagnosed diabetic subjects demonstrated 'normalalbuminuria' while established diabetic subjects were 'microalbuminuric,' consistent with the hypothesis that persistent hyperglycaemia is associated with early diabetic nephropathy. Furthermore, in the present study increased



levels of lipid peroxidation and decreased antioxidants/defence mechanisms were noted in the established diabetic group, again suggesting a link between persistent hyperglycaemia, poor metabolic control including dyslipidaemia, oxidative stress and an increased risk of developing microalbuminuria. Several other studies have also suggested that oxidative stress, as a result of poor glycaemic control and subsequent tubular and glomerular insult proceeding in parallel with endothelial cell damage may precede cellular damage and hence the development of diabetic nephropathy (Yaqoob *et al*, 1994; Skrha and Hilgertova, 1999). Early tubular insult characterised by NAG enzymuria (Whiting *et al*, 1979; UKPDS, 1993; Wetigasser *et al*, 1999) which occurs prior to microalbuminuria have also been described. The present study has clearly demonstrated that increased NAG enzymuria and TBARS excretion, in the absence of abnormal levels of microalbuminuria, were observed in recently diagnosed diabetic subjects, again supporting the concept that oxidative damage and renal tubular insult may accompany or even precede the onset/presence of microalbuminuria (Yaqoob *et al*, 1994).

Furthermore, in both diabetic group, the presence of enhanced oxidative stress, associated with increased lipid peroxidation and depletion of antioxidants was associated with elevation of both  $\alpha$ GST and  $\pi$ GST enzymuria. This is an important observation as pathological levels of microalbuminuria were not present in the recently diagnosed diabetic group and GST estimation could provide further evidence identifying those individuals at risk of developing diabetic renal dysfunction. Furthermore, as GSTs are important cellular components of the defence mechanisms responsible for the metabolism of lipid peroxides and the products of oxidative stress, any reduction in their cellular

concentration could further enhance any oxidative stress present. The results of the present study do inevitably suggest an involvement of GST in the pathogenesis of oxidative stress as a result of persistent hyperglycaemia and enhanced lipid peroxidation in the renal tubules. However, these suggestions would require further experimental validation.

One speculative possible explanation for the increased GST enzymuria present in both diabetic groups could be that the increased enzymuria reflects an increase in cellular activities of this antioxidant defence mechanism as a compensatory consequence of increased lipid peroxidation. However, this again would also require experimental validation as does the association of GSTs with increased lipid peroxidation, enhanced oxidative stress and the role of apoptosis in diabetic nephropathy as suggested recently by several research groups (Zhang *et al*, 1997; Kannan and Jain, 2000).

## **6.5 Conclusions**

Although the exact pathophysiological processes underlying diabetic nephropathy remain unclear, evidence is now accumulating linking free radical formation and oxidative stress to the cascade of events leading to diabetic renal. The present and several other studies have shown that persistent hyperglycaemia associated with poor metabolic control, particularly dyslipidaemia, is the major determinant in both the initiation and development of early diabetic nephropathy (DCCT, 1993; DCCT, 1995; UKPDS, 1998). Not surprisingly, persistent hyperglycaemia is temporally related to other biochemical

mechanisms suggested to play a role in early diabetic nephropathy, for example glucose autooxidation, non-enzymatic protein glycation, polyol pathway and altered lipoprotein metabolism resulting ultimately in the generation of free radicals. The present studies have indeed demonstrated an association between glycaemic control and glycation products, dyslipidaemia and the generation of free radicals. In addition, these studies have also demonstrated reduced both total antioxidant status and antioxidant defence mechanisms associated with renal glomerular and tubular dysfunction and consistent with enhanced oxidative stress being present in the diabetic state.

In addition the present studies have investigated sensitive biomarkers including circulating cystatin C, microalbuminuria and sialic acid concentrations, indicators glomerular function and/or permeability, LMWP and enzymuria, including NAG, AAP and both  $\alpha$ GST and  $\pi$ GST. The results obtained clearly demonstrate the potential for measurements of cystatin C, sialic acid and enzymuria, particularly  $\alpha$ GST and  $\pi$ GST, to provide a significant and effective means for identifying those diabetic subjects at risk of developing early renal changes, particularly before the development of microalbuminuria. The results obtained here are particularly gratifying considering the apparent insensitivity of some of the standard measures regularly and routinely used currently in diabetic clinics, for example serum/plasma creatinine concentrations and some methods for quantifying microalbuminuria, the earliest indicator of incipient nephropathy in diabetics (Viberti, 1982; Mogensen, 1984; Okada *et al*, 1996). However, this would involve assessing diabetic patients more regularly in the clinic than at present and there is an obvious resource implication.



While the mechanisms involved in the development of diabetic nephropathy remain difficult to demonstrate in the clinical situation, especially considering the natural history of the disease process, the studies presented here have indicated that the measurement of sensitive biomarkers can indicate perhaps either the presence of, or an increased magnitude of, some of the implicated processes. For example, enzymuria can be linked to site specific insult within the renal tubular system in a similar fashion as plasma sialic acid can indicate loss of glomerular polyanion, total antioxidant status a reflection of the potential of oxidative stress, microalbumuria an increase in glomerular permeability and cystatin C, changes in GFR reflecting either hyper or hypofiltration at the glomerulus. Furthermore, while this study has demonstrated that renal functional changes could be associated with increased lipid peroxidation, oxidative stress and reduced antioxidants, it also provides a laboratory basis for assessing corrective therapy for the above, for example Vitamin E and selenium dietary supplementation to maximise protection against oxidative damage. Consequently, and similar to the findings of Koya *et al*, (2003) this study has concluded that free radical mediated oxidative stress may play an important role in the development and progression of diabetic nephropathy and antioxidant treatment would be potential corrective therapeutic possibility.

## 6.6 Future work

The present study clearly demonstrated that free radical mediated oxidative stress plays a crucial role in the pathogenesis of diabetic nephropathy. The biomarkers for free radical damage are hampered by several factors. The measurement of free radical activity is difficult due to several factors including lack of specificity and sensitivity, difficulty in the definition of commonly accepted normal reference intervals, in part because several methods can be used for the same oxidative parameters and finally, effective control materials for antioxidants and oxidative stress parameters are not commercially available. Consequently, precise analytical methods for oxidative stress biomarkers with improved sensitivity and specificity need to be introduced for application in the routine clinical laboratory that will allow improved interpretation of laboratory results to be achieved. Although measurement of AGEs and *N*- $\epsilon$ -(carboxymethyl) lysine (CML) are technically difficult, suitable routine analytical methods have still to be developed.

Dietary supplementation with antioxidants, such as vitamin E and reduced glutathione, may be advantageously used in subjects with diabetes. Antioxidants might reduce protein glycosylation, inhibit glucose autoxidation and the formation of AGEs, while at the same time, operate as scavengers for free radicals, generated by both glucose autoxidation and glycated proteins (Ceriello and Giugliano, 2000). However, long-term intervention studies are now needed to evaluate the efficacy of this new therapeutic approach in the prevention and treatment of diabetic nephropathy. This would also allow further insight

into the relationship between hyperglycaemia, protein glycation, AGEs, total antioxidant status and lipid peroxidation events.

Although the natural history of diabetic nephropathy is well known and new sensitive non-invasive biomarkers have been developed for the early detection of renal insult, the early detection of diabetic kidney damage has been neglected particularly the contribution that tubular function makes to overall renal function (Whiting and Price, 2001). Consequently, longitudinal large prospective studies are needed now in order to further evaluate the predictive value of both proximal and distal tubular insult in the development of diabetic nephropathy.

The role of free radicals and a relationship between both renal function and structure would be further examined by the use of animal models of diabetes (Streptozotocin induced diabetes or the use of BB-Wistar rats). At various time points following either the recognition/induction of diabetes, PT cells would be isolated and the generation of free radicals assessed. Renal histology and function as well as estimation of glycaemia and protein glycation would also be estimated allowing any temporal relationship between lipid peroxidation, renal function and histological changes to be established. In addition, renal mitochondria could be prepared from either human or animal kidney and oxidative metabolism investigated in the presence of high glucose concentrations. This could include estimates of free radical formation, ATP/ADP and NADH/NAD ratios.



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